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14. ABSTRACT

The research outlined in this proposal is aimed directly at improving the effectiveness and duration of endocrine therapies.
Our approach builds upon our initial observations that tamoxifen up-regulates 14-3-3ζ, a key scaffold protein that is associated with poor outcome of patients on tamoxifen endocrine therapy. 14-3-3ζ interacts with and enhances the activity of growth factor receptors and kinases that are overexpressed in breast cancers that are resistant to endocrine therapies. The experiments outlined are aimed at validating 14-3-3z as a marker for risk of recurrence due to the development of endocrine resistance and at establishing this protein as a target whose inhibition would enhance the effectiveness of endocrine therapies, by maintaining endocrine sensitivity. Thus, the outcome of this research has the potential for improving the selection of breast cancer patients most likely to benefit from endocrine therapies and provide a new avenue for enhancing the effectiveness of these therapies for treatment of breast cancer.

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INTRODUCTION

Endocrine therapies (antiestrogens such as tamoxifen or aromatase inhibitors such as letrozole) initially benefit many of the ca. 75% of breast cancers that are estrogen receptor positive. The effectiveness of endocrine therapies, however, is often lost with time because the tumor cells become resistant. We have found that loss of benefit from endocrine therapy involves increased activity of protein kinases and growth factors that work along with the protein 14-3-3ζ (zeta) to promote survival of the tumor cells. Our studies indicate that high levels of the 14-3-3ζ protein are found in breast cancers that show a poor clinical outcome on endocrine therapy. These findings imply that targeting 14-3-3ζ might prove useful for enhancing and prolonging the effectiveness of endocrine therapies.

BODY

Our work during this DOD grant has revealed that 14-3-3ζ is a predictor of early time to recurrence and distant metastasis in hormone receptor-positive and -negative breast cancers. The 14-3-3ζ gene, on 8q22, is often amplified in breast cancer. It encodes a survival factor that interacts with and stabilizes many key signaling proteins, thereby contributing to therapy resistance and poor clinical outcome [R1]. In the first year of this grant we have shown that high expression of 14-3-3ζ in ER-positive breast cancers was associated with a poor clinical outcome for women on tamoxifen [R 1 and A1]. We have now found that reducing cellular levels of 14-3-3ζ markedly increases apoptosis of breast cancer cells, reduces cell proliferation and motility, decreases receptor tyrosine kinase signaling and, importantly, reverses antiestrogen resistance, thereby rendering endocrine-resistant breast cancer cells sensitive to antiestrogens . As expected, these beneficial effects of 14-3-3ζ depletion were lost upon re-expression of 14-3-3ζ (Fig. 1).

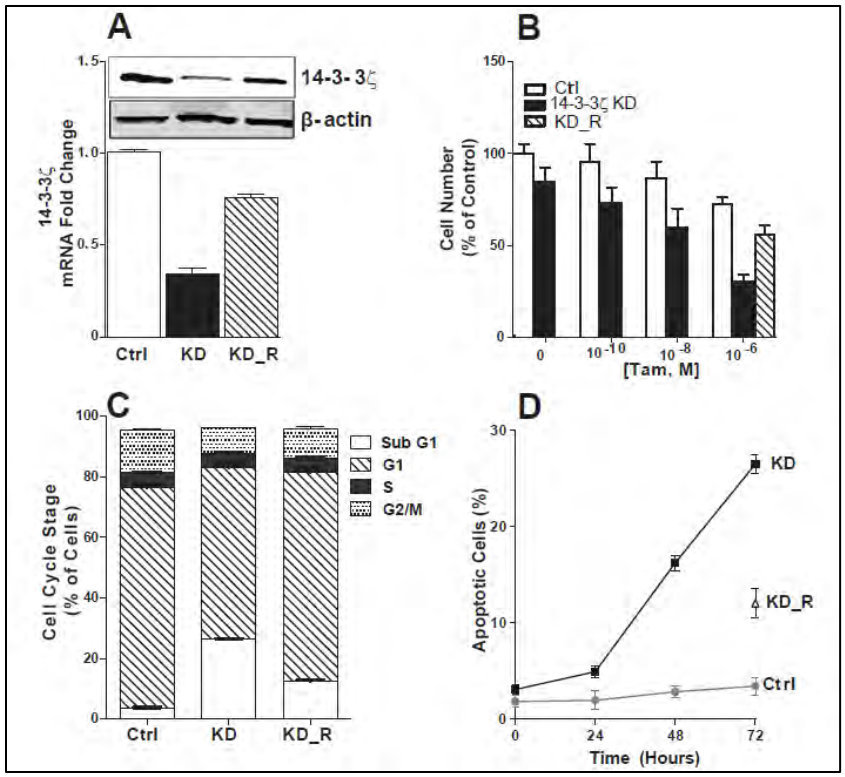


Figure 1 **Characterization of the phenotypic properties of MCF-7 cells with knockdown of 14-3-3ζ**. (a) 14-3-3ζ RNA and protein levels were evaluated by quantitative PCR and western blot in cells stably expressing control shRNA (Ctrl) or 14-3-3ζ shRNA knockdown (KD) and in KD cells transfected with wild type 14-3-3ζ (KD_R, knockdown and reexpression). (b) Cell viability in response to different concentrations of tamoxifen (Tam) for 48 hours for Ctrl or 14-3-3ζ KD or KD_R cells. Cell number for vehicle-treated control cells is set as 100%. (c) Percentage of cells in the different cell cycle stages for Ctrl and 14-3-3ζ KD or KD_R cells treated with 1 μM Tam for 72 hours. (d) Percentage of apoptotic cells in Ctrl, 14-3-3ζ KD, and KD_R cells treated with 1 uM Tam.

Further, in four independent breast cancer microarray data sets from over 400 women, we found that high levels of 14-3-3ζ were associated predominantly with the ER-positive HER2 expressing luminal B subtype of breast cancers, and with a poor prognosis. Moreover, high expression of 14-3-3ζ correlated strongly with over-expression of genes functioning in mitosis and cytokinesis, including Aurora Kinase B, Polo Kinase 1, BIRC5 (survivin), and FOXM1. The latter four proteins were significantly decreased upon reduction of cellular 14-3-3ζ, suggesting their coregulation.

In the second year of this DOD postdoctoral fellowship grant, we have revealed that the tamoxifen up-regulation of 14-3-3ζ results from its ability to rapidly down-regulate a microRNA, miR-451, that specifically

targets 14-3-3ζ [Publication 1]. The levels of 14-3-3ζ and miR-451 were inversely correlated, with 14-3-3ζ being elevated and miR-451 being at a greatly reduced level in tamoxifen-resistant breast cancer cells. Of note, down-regulation of miR-451 was selectively elicited by tamoxifen but not by other SERMs such as raloxifene or ICI182,780 (Fulvestrant) (Fig. 2).

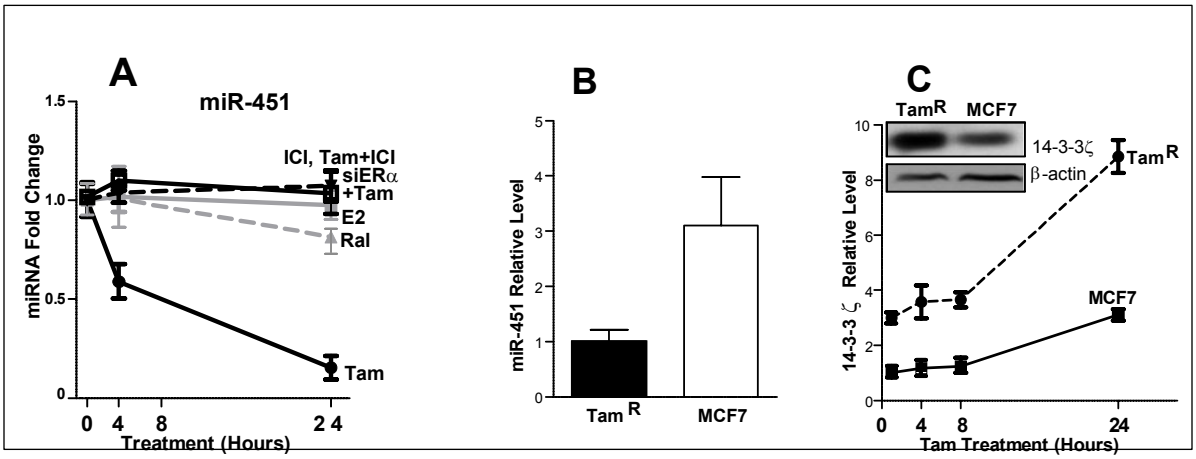


Fig.2 miR-451 is downregulated by tamoxifen in ER-positive breast cancer cells and its expression is inversely correlated with 14-3-3ζ in MCF7 and tamoxifen-resistant (TamR) cells. a) Regulation of miR-451 in MCF7 cells by the estrogen receptor ligands Tamoxifen (Tam), Raloxifene (Ral), estradiol (E2) and ICI 182,780 (ICI Fulvestrant), or after 0, 4 and 2 h of treatment. ERα was also depleted from cells by treatment with siRNA for ERα before Tam treatment for 0, 4 and 24h. (b) Levels of miR-451 in MCF7 and TamR breast cancer cells detected by quantitative PCR. (c) Change in 14-3-3ζ and miR-451 levels analyzed by quantitative RT–PCR in MCF7 and TamR cells after treatment with Tam. (From publication 1)

Increasing the level of miR-451 by overexpression, which decreased 14-3-3ζ, suppressed cell proliferation and colony formation, markedly reduced activation of HER2, EGFR, and MAPK signaling, increased apoptosis, and importantly, restored the growth inhibitory effectiveness of SERMs in endocrine-resistant cells. Opposite effects were elicited by miR-451 knock-down. Thus, we identify tamoxifen down-regulation of miR-451, and consequent elevation of the key survival factor 14-3-3ζ, as a mechanistic basis of tamoxifen-associated development of endocrine resistance [Publications 1-2, A1, A4] (Fig 3).

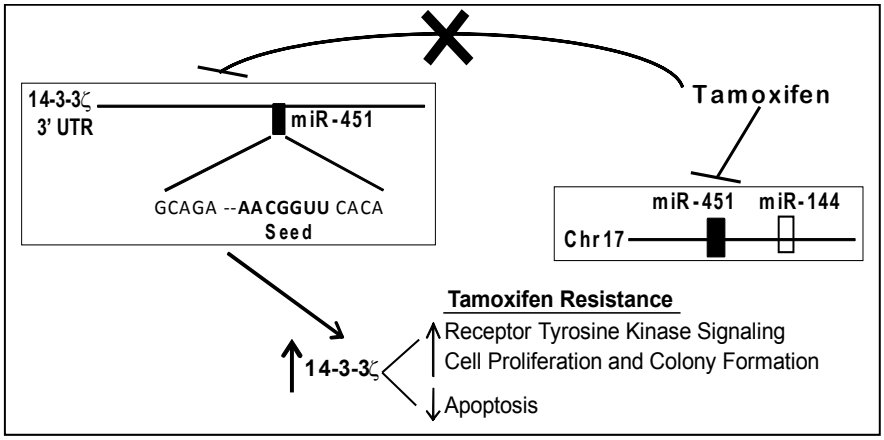
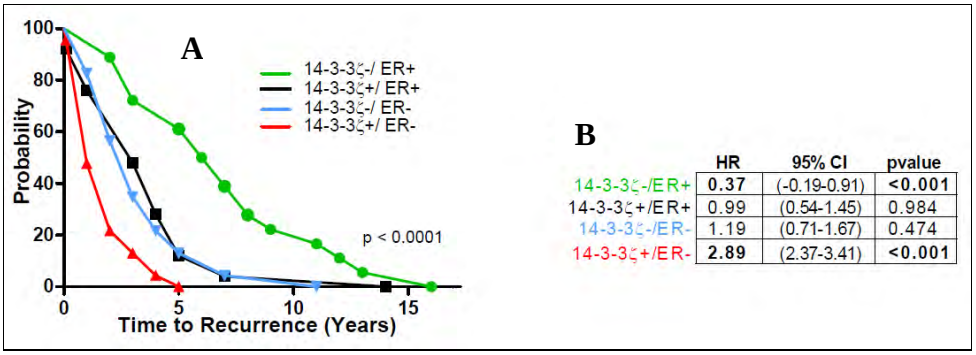


Fig.3 **Schematic representation of the effect of tamoxifen on miR-451 and 14-3-3ζ regulation and their impact on breast cancer cell phenotypic properties leading to tamoxifen resistance.** Tamoxifen downregulates miR-451, resulting in the upregulation of 14-3-3ζ, with consequent increased receptor tyrosine kinase signaling, increased cell proliferation and colony formation, and reduced apoptosis, thereby leading to tamoxifen resistance. (From publication 1)

These findings suggest that therapeutic approaches to increase expression of this tumor suppressor-like microRNA should be considered to down-regulate 14-3-3ζ and enhance the effectiveness of endocrine therapies. Furthermore, the selective ability of the SERM tamoxifen but not raloxifene to regulate miR-451 and 14-3-3ζ may assist in understanding differences in their activities, as seen in the STAR breast cancer prevention trial and in other clinical trials.

In light of the discvery we have made in year 1 and 2 in year 3 we report the relationship between the expression of 14-3-3ζ, estrogen receptor (ERα), FOXM1, and other molecular markers in matched primary and recurrence tumor tissue from women with breast cancer and how these impact time to recurrence, properties of the recurrent tumors, and site of metastasis [MS1 and A7].

In this cohort of 130 patients selected for this study because all experienced disease recurrence, median time to recurrence 3 years (range 1-17 yrs), of whom ca. 55% were ERα-positive, our analysis of primary tumor microarray cores revealed that primary tumors that were 14-3-3ζ-positive and ERα-negative had the earliest time to recurrence (median time of recurrence1yr, p <0.001, hazard ratio of 2.89 (95% CI of 2.37-3.41), while median time to recurrence was 3 yrs for 14-3-3ζ-negative and ER-negative tumors and 14-3-3ζ-positive and ER-positive tumors, and 7 yrs for 14-3-3ζ-negative and ER-positive tumors (Fig. 4).



B

	HR	95% CI	pvalue
14-3-3ζ-/ER+	0.37	(-0.19-0.91)	<0.001
14-3-3ζ+/ER+	0.99	(0.54-1.45)	0.984
14-3-3ζ-/ER-	1.19	(0.71-1.67)	0.474
14-3-3ζ+/ER-	2.89	(2.37-3.41)	<0.001

Fig.4 (A) Kaplan Meyer analysis showing time to recurrence (p value <0.0001). (B) Hazard ratios (HR) and 95% confidence intervals and p values are shown for each cluster group.

To assess the prognostic importance of 14-3-3ζ, we stratified primary tumors based on 14-3-3ζ IHC and compared these to levels in the matched recurred tumors. Of recurred tumors, 70-75% were positive for 14-3-3ζ, up from the ca. 50% positivity of the primary tumors. Thus, a significant proportion of primary tumors low/negative for 14-3-3ζ became positive in the recurrence (p=0.0001). Positive 14-3-3ζ status was correlated with site of recurrence, and showed a propensity for metastases to lung and chest wall. Multifactor correlation regression analysis revealed 14-3-3ζ status to be a non-redundant (i.e. independent) variable that adds clinical strength in predicting recurrence in ER-positive and -negative breast cancers and provides information beyond that of all other clinical pathological features examined (tumor size, grade, receptor status, FOXM1, CERB2, EGFR, p53, positive lymph nodes).

Thus, high expression of 14-3-3ζ in the primary tumor or its acquisition in the recurrence was significantly associated with earlier time to recurrence and with distant metastasis in both ER-positive and ER-negative breast cancers [MS1 and A7].

As previously stated, in year 1 and 2 of this DOD grant we have identified a gene signature (29 genes) associated with high 14-3-3ζ levels in breast tumors [Publications 1, 2, A1, A4, A7]. These encompassed many with functions in mitosis and cytokinesis, including aurora kinase-B, polo-like kinase-1, CDC25B, and BIRC5/survivin. This gene signature correlated with early recurrence and risk of metastasis, and was found predominantly in luminal B breast cancers, the more aggressive ER-positive molecular subtype. The expression of the signature genes was significantly decreased or increased upon reduction or overexpression of 14-3-3ζ in ER-positive breast cancer cells, indicating their coregulation. 14-3-3ζ was also found to play a critical role in the regulation of FOXM1, with 14-3-3ζ acting upstream of FOXM1 to regulate cell division-signature genes. Depletion of 14-3-3ζ markedly increased apoptosis, reduced proliferation and receptor tyrosine kinase (HER2 and EGFR) signaling, and, importantly, reversed endocrine resistance, thereby rendering endocrine-resistant breast cancer cells sensitive to antiestrogens [Publications 1, A1, A4].

Because we found that 14-3-3ζ regulates the expression of FOXM1, we investigated the status of FOXM1 in tumors, and we observed that FOXM1 was significantly correlated with 14-3-3ζ positivity (p=0.042), and tumors positive for both factors showed the highest risk for early recurrence (Fig 2). Thus, positivity for FOXM1 improves the value of 14-3-3ζ as a predictor of time to recurrence [MS1 and A7].

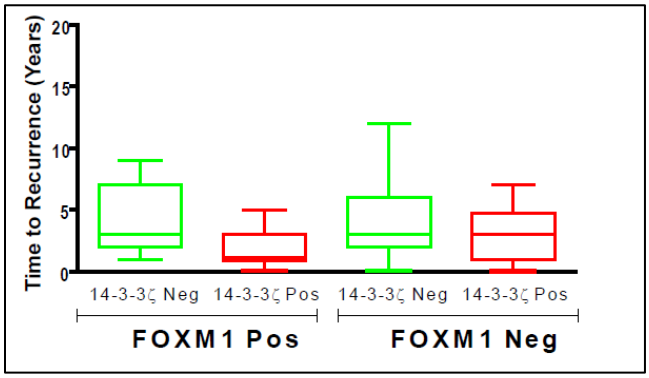


Fig.2 Tumors were stratified by FOXM1 and 14-3-3ζ immunohistochemistry status. Neg denotes low-negative; pos denotes high protein expression. Box plots show time to recurrence (mean ± SD).

FOXM1 has long been known to be associated with mitosis [R2], but it has additional functions including regulation of the mitotic spindle assembly checkpoint, thereby ensuring proper chromosome stability and segregation during mitosis [R3]. A key role in metastasis has also been revealed more recently [4]. Our findings underscore the very detrimental roles played by both 14-3-3ζ and FOXM1 in tumor aggressiveness and metastasis, and suggest that reducing their expression or interfering with their actions might substantially improve the clinical outcome for breast cancer patients.

Although there has been some success in the development of inhibitors for 14-3-3 proteins [R4], achieving selectivity for specific family members has proven to be challenging, such that selective inhibitors for 14-3-3ζ have not yet been developed. We believe that approaches aimed at intercepting downstream effector targets of 14-3-3ζ, such as FOXM1, might prove to be of benefit in overcoming therapeutic resistance. Therefore, for inhibition of FOXM1, we used a cell-permeable WT ARF 26-44 peptide and control mutant ARF peptide 37-44 with nine D-Arg residues at the N-terminus to increase cell uptake of the peptide and showed decrease in cell

proliferation characterized by reducing the levels of FOXM1 cell-cycle target genes like Aurora kinase B, PLK1 and activation of MAPK. Interestingly, ARF but not the mutant peptide decreased significantly the levels of 14-3-3ζ suggesting that the use of this inhibitor may be proven useful to block 14-3-3ζ signaling (Fig.3).

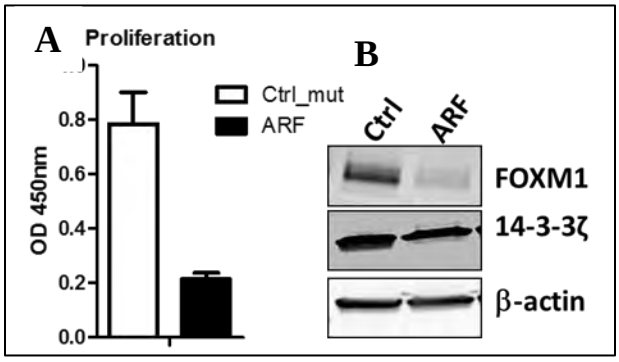


Fig.3 (A) Growth of MCF7 cells in the presence of FOXM1 inhibitor (ARF) was measured after 6 days. (B) Western blots of whole cell extracts from MCF7 cells in the presence of FOXM1 inhibitor (ARF) for 3 days and probed with antibodies against FOXM1 14-3-3ζ. β-actin was used as loading control.

By immunofluorescence confocal microscopy, we observe that 14-3-3ζ cellular localization is essential during different stages of cell mitosis and cytokinesis. Further, we found that 14-3-3ζ interaction with microtubule associated proteins interferes with the response to microtubule-targeted agents thereby suggesting a key role of 14-3-3ζ to chemotherapy resistance and poor clinical outcome [MS2].

Indeed, there is much evidence that forkhead factors bind to chromatin and affect chromatin accessibility and activity. It is increasingly appreciated through our work and that of others that nuclear and extranuclear-initiated actions of estrogens and antiestrogens are crucial in regulation of estrogen receptor activity on gene expression, cell proliferation, and cell survival, and we believe that ERKs, along with 14-3-3ζ and FOXM1, collaborate in controlling the phenotypic properties of breast cancer cells.

FOXM1 resides mainly within the nucleus, with much of the nuclear-localized protein being chromatin associated. Partially Its activation is dependent on hormone treatment and our recent studies examining FOXM1 cistrome (chromatin binding sites) across the genome after treatment with estradiol and tamoxifen have highlighted the enrichment of binding of the transcription factor to the promoter regions of specific genes that are involved in proliferation and phenotypic behavior of breast cancer stem cells. Taking advantage of ERK2 cistrome data available from our laboratory and of others publicly available we have examined the interplay between FOXM1 and the MAPK pathway in hormone-resistant breast cancers. Results from this analysis showed the importance of cooperation between ERK2, and FOXM1 at the chromatin level in regulating gene expression programs that control the development of a more aggressive breast cancer phenotype [MS3].

Taking together, our data indicate that 14-3-3ζ can serve as a marker of therapy resistance and as a key therapeutic target for endocrine therapy sensitization and effective tumor suppression. Resistance to endocrine therapies is associated with elevated levels of 14-3-3ζ and FOXM1, and enhanced signaling through growth factor receptor and downstream kinase pathways including MAPK. Further, these signaling cascades result in

the activation of additional kinases such as polo-like kinase 1 and the cyclin-CDKs, which are part of the 14-3-3ζ gene signature and are regulated at the chromatin levels by FOXM1.

Significance

Our goal is to improve the effectiveness of endocrine therapies in breast cancer by reducing or reversing endocrine resistance. Our research has revealed that 14-3-3ζ is a key predictive marker for risk of failure on endocrine therapy and early time to recurrence. It does this by playing a pivotal role impacting growth factor signaling and promoting breast cancer cell survival and resistance to therapies. Targeting 14-3-3ζ and its coregulated proteins, such as FOXM1, are being explored by us in this DOD project to enhance the effectiveness of endocrine and chemo-therapies and reduce risk of breast cancer recurrence, thereby making treatments more effective for many breast cancer patients.

KEY RESEARCH ACCOMPLISHMENTS

On this projected funded by the DoD we were able to identify that:

1. High levels of 14-3-3ζ were associated predominantly with the ER-positive HER2 expressing luminal B subtype of breast cancers, and with a poor prognosis.
2. High expression of 14-3-3ζ correlated strongly with over-expression of genes functioning in mitosis and cytokinesis.
3. Reducing cellular levels of 14-3-3ζ markedly increases apoptosis of breast cancer cells, reduces cell proliferation and motility, decreases receptor tyrosine kinase signaling and, importantly, reverses antiestrogen resistance.
4. Tamoxifen regulation of 14-3-3ζ is mediated by a deregulation of miR-451.
5. Tamoxifen downregulation of miR-451, and consequent elevation of the key survival factor 14-3-3ζ , is a mechanistic basis of tamoxifen-associated development of endocrine resistance.
6. Aggressive metastatic breast cancers show higher 14-3-3ζ protein levels, with alterations in intracellular localization, compared to more indolent breast cancers.
7. Tamoxifen induces a specific FOXM1 cistrome regulating genes involved in proliferation and cancer stem cells phenotypic behaviors.
8. 14-3-3ζ is associated with chemotherapy resistance by regulating microtubules associate proteins

Reportable Outcomes

Published Abstracts from this project:

A1. Bergamaschi, A., Frasor, J., and Katzenellenbogen, B.S., A Gene Signature and Molecular Phenotype Associated with High Expression of 14-3-3ζ and Its Correlation with Antiestrogen Resistance in Breast Cancer. 2nd Jensen Symposium on Nuclear Receptors, University of Cincinnati Medical Center, Cincinnati, OH, October 2009.

A2. Katzenellenbogen, B.S., Madak-Erdogan, Z., Bergamaschi, A., Stossi, F., Lupien, M., Brown, M., Katzenellenbogen, J.A. Genomics of Estrogen Receptor Signaling in Breast Cancer and Endocrine Resistance. Keystone Symposium on Nuclear Receptors: Signaling, Gene Regulation and Cancer/Nuclear Receptors: Development, Physiology and Disease, Keystone, Colorado, March, 2010

A3. Katzenellenbogen, B.S., Madak-Erdogan, Z., Bergamaschi, A., Stossi, F., Charn, T.H. Genomics of Estrogen Receptor Signaling in Target Cells. Frontiers in Estrogens, SERMs, and TSEC Scientific Meeting, Philadelphia, PA, April, 2010

A4. Bergamaschi, A., and Katzenellenbogen, B.S., Mechanistic Basis of Tamoxifen Associated Development of Endocrine Resistance in Breast Cancer, AACR, Orlando, FL, April 2011.

A5. Katzenellenbogen, B. S., Madak-Erdogan, Z., Stossi, F., and Bergamaschi, A., Integration of cell signaling in the genomic actions of estrogen receptors, Estrogens, SERMS and TSECs Meeting, Clearwater, FL, April, 2011.

A6. Katzenellenbogen, B. S., Madak-Erdogan, Z., Bergamaschi, A., Stossi, F., and Charn, T. H., Genomics of Estrogen Receptor Signaling in Target Cells, ERβ Symposium "Therapeutic potential of ERβ as drug target", Stockholm, Sweden, May, 2011.

A7. Bergamaschi, A., Frasor, J., Borgen, K., Stanculescu, A., Johnson, P., Rowland, K., Wiley, E.L., and Katzenellenbogen, B.S., Identification of 14-3-3z as a predictor of early time to recurrence and a molecular target in metastatic hormone receptor-positive and -negative breast cancers. AACR 103rd Annual Meeting, Chicago, March 29-April 2, 2012.

A8. Holton, S.E., Bergamaschi, A., Katzenellenbogen, B.S., and Bhargava, R., A spectroscopic signature associated with hormone sensitivity in 3D co-culture models of breast cancer. AACR 103rd Annual Meeting, Chicago, March 29-April 2, 2012.

A9. Madak- Erdogan, Z., Bergamaschi, A., Ventrella, R., Lu, H., Katzenellenbogen, B.S., Estrogen Receptor Regulation of ERK5 and Cofilin Localization Impacts Breast Cancer Phenotypic Properties and Endocrine Resistance. ENDO 2012: The 94th Annual Meeting & Expo, Houston, TX, June 2012.

Publications from this project:

1. Bergamaschi, A., Katzenellenbogen, B.S. Tamoxifen Down-Regulation of miR-451 Increases 14-3-3ζ and Promotes Breast Cancer Cell Survival and Endocrine Resistance. Oncogene. 2011 Jun 13. (Appendices 1)

2. Bergamaschi, A., Christensen, B., Katzenellenbogen, B.S. Reversal of Endocrine Resistance in Breast Cancer: Interrelationships Among 14-3-3ζ, FOXM1, and a Gene Signature Associated with Mitosis. Breast Cancer Res. 2011 Jun 29;13(3):R70. (Appendices 2)

Manuscript in preparation from this project:

MS1. Bergamaschi, A., Frasor, J., Borgen, K., Stanculescu, A., Johnson, P., Rowland, K., Wiley, E. L., and. Katzenellenbogen, B. S. 14-3-3ζ as a Predictor of Early Time to Recurrence and Distant Metastasis in Hormone Receptor-Positive and -Negative Breast Cancers. To be submitted

MS2. Bergamaschi, A., Madak-Erdogan, Z., and Katzenellenbogen, B.S. 14-3-3ζ Contributes to Resistance to Microtubule-Targeted Agents by Regulating Microtubule Associated proteins.

MS3. Bergamaschi, A., Madak-Erdogan, Z., Lu H. and Katzenellenbogen, B.S. Tamoxifen resistance FOXM1 cistrome is associated with cell proliferation and phenotypic behavior of breast stem cancer cells.

Conclusion

Our studies indicate that 14-3-3ζ is a major contributor to endocrine resistance and to breast cancer aggressiveness and recurrence. Our proposed studies should clarify the role of 14-3-3ζ by analysis of tumor tissue microarrays and characterization of gene expression signatures. We will also explore several therapeutic strategies to block the actions of 14-3-3ζ or reduce its levels, so as to maintain or restore endocrine sensitivity in breast cancer, thereby making endocrine therapies more effective for many breast cancer patients.

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Reportable Outcomes

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A7. **Bergamaschi, A.**, Frasor, J., Borgen, K., Stanculescu, A., Johnson, P., Rowland, K., Wiley, E.L., and Katzenellenbogen, B.S., Identification of 14-3-3z as a predictor of early time to recurrence and a molecular target in metastatic hormone receptor-positive and -negative breast cancers. AACR 103rd Annual Meeting, Chicago, March 29-April 2, 2012.

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Oncogene (2011) 1–9

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ORIGINAL ARTICLE

Tamoxifen downregulation of miR-451 increases 14-3-3ζ and promotes breast cancer cell survival and endocrine resistance

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Many estrogen receptor (ER)-positive breast cancers respond well initially to endocrine therapies, but often develop resistance during treatment with selective ER modulators (SERMs) such as tamoxifen. We have reported that the 14-3-3 family member and conserved protein, 14-3-3ζ, is upregulated by tamoxifen and that high expression correlated with an early time to disease recurrence. However, the mechanism by which tamoxifen upregulates 14-3-3ζ and may promote the development of endocrine resistance is not known. Our findings herein reveal that the tamoxifen upregulation of 14-3-3ζ results from its ability to rapidly downregulate microRNA (miR)-451 that specifically targets 14-3-3ζ. The levels of 14-3-3ζ and miR-451 were inversely correlated, with 14-3-3ζ being elevated and miR-451 being at a greatly reduced level in tamoxifen-resistant breast cancer cells. Of note, downregulation of miR-451 was selectively elicited by tamoxifen but not by other SERMs, such as raloxifene or ICI182,780 (Fulvestrant). Increasing the level of miR-451 by overexpression, which decreased 14-3-3ζ, suppressed cell proliferation and colony formation, markedly reduced activation of HER2, EGFR and MAPK signaling, increased apoptosis, and, importantly, restored the growth-inhibitory effectiveness of SERMs in endocrine-resistant cells. Opposite effects were elicited by miR-451 knockdown. Thus, we identify tamoxifen downregulation of miR-451, and consequent elevation of the key survival factor 14-3-3ζ, as a mechanistic basis of tamoxifen-associated development of endocrine resistance. These findings suggest that therapeutic approaches to increase expression of this tumor suppressor-like miR should be considered to downregulate 14-3-3ζ and enhance the effectiveness of endocrine therapies. Furthermore, the selective ability of the SERM tamoxifen but not raloxifene to regulate miR-451 and 14-3-3ζ may assist in understanding differences in their activities, as seen in the STAR (Study of Tamoxifen and Raloxifene) breast cancer prevention trial and in other clinical trials.
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Introduction

Tamoxifen has proven to be one of the most successful agents in the management of estrogen receptor (ER)-positive breast cancers, and it has been a mainstay of endocrine therapy for breast cancer over the last 30 years. Large studies have shown its value in the improvement of survival in early breast cancer (Osborne, 1998; EBCTCG, 2005), as well as an improvement in the quality of life for patients with metastatic disease (Jaiyesimi *et al*., 1995). However, despite the remarkable benefits of using ER-targeted therapies, including selective ER modulators (SERMs) such as tamoxifen in the *ca* 70% of women with hormone-sensitive breast cancers expressing ERs, many will eventually experience disease progression, owing to the development of resistance to the therapy. The mechanisms that underlie endocrine resistance are complex and are not fully understood, despite significant advances in defining some of the players involved (Creighton *et al*., 2006; Green and Carroll, 2007; Arpino *et al*., 2008; Massarweh *et al*., 2008).

In a previous study, based on genome-wide gene expression analyses in human breast tumors and in ER-positive breast cancer cells, we found 14-3-3ζ (also known as YWHAZ) to be upregulated by tamoxifen and observed a poor clinical outcome on tamoxifen treatment for patients with high levels of 14-3-3ζ in their breast tumors (Frasor *et al.,* 2006). This correlation between overexpression of 14-3-3ζ and early onset of recurrence was also observed by us in data from other large microarray gene expression studies, implying 14-3-3ζ to be a marker of poor prognosis in women with ER-positive breast cancers (Bergamaschi *et al*., 2009).

14-3-3ζ is a member of the highly conserved family of seven 14-3-3 proteins, all encoded by different genes (Tzivion *et al*., 2006). 14-3-3ζ serves as a pivotal factor that binds and stabilizes key proteins involved in signal transduction, cell proliferation and apoptosis (Ando *et al*., 2004; Costa, 2005), including EGFR, HER2, PKC, β-catenin and RAF-1 (McPherson *et al*., 1999; Oksvold *et al*., 2004; Filipowicz *et al*., 2008).

In view of the very detrimental role of 14-3-3ζ in promoting cancer cell survival and endocrine resistance, we were interested in understanding how 14-3-3ζ is regulated, and in particular, how cellular levels of 14-3-3ζ might be downregulated to enhance responsiveness to endocrine therapies. As growing evidence links microRNAs (miRs) to the control of many crucial processes in cancer, including proliferation, differentiation and apoptosis (Croce, 2009), we have herein explored the involvement of miRs in the regulation of 14-3-3ζ. The great importance of miRs is underscored by the fact that they are estimated to have roles in regulating the expression of more than one-third of all human genes. miRs, which are small, *ca* 22-nucleotide RNA sequences, bind principally to the 3′-untranslated region (UTR) region of mRNAs of protein-coding target genes to direct their repression by destabilizing target mRNAs and decreasing mRNA translational efficiency (Bartel, 2004; Harfe, 2005; Guo *et al*., 2010).

In the present study, to investigate the mechanism underlying tamoxifen upregulation of 14-3-3ζ, we first identified, by bioinformatic analysis, potential miRs that might regulate 14-3-3ζ, and then went on to show tamoxifen downregulation of miR-451 and to delineate the role of miR-451 in regulating 14-3-3ζ and its impact on the aggressiveness, cell survival properties and endocrine sensitivity of ER-positive breast cancer cells. The findings suggest that increasing the cellular level of miR-451 might be a useful therapeutic approach for reversing endocrine resistance and enhancing the efficacy of endocrine therapies in the >40% of breast tumors that overexpress 14-3-3ζ.

Results

Tamoxifen downregulates miR-451 and there is an inverse relationship between miR-451 and 14-3-3ζ levels

Although we have reported 14-3-3ζ to be upregulated by the antiestrogen (SERM) tamoxifen in breast cancer (Frasor *et al*., 2006), the mechanism regulating tamoxifen-driven stimulation of 14-3-3ζ is not known. Given the growing evidence for the importance of miRNAs in regulating gene expression, we envisioned that miRNAs might have an important role in modulating 14-3-3ζ expression. Using two different target prediction algorithms, TargetScan (http://www.targetScan.org/) and miRanda (http://www.microrna.org/microrna/home.do), we found nine miRNAs that were predicted by both algorithms to target 14-3-3ζ. From these, we chose to investigate miR-451 because it showed relatively few potential targets, and 14-3-3ζ was the only predicted target from the 14-3-3 family. We then examined whether tamoxifen might regulate miR-451. As shown in Figure 1a, where we monitored the effects of different ER ligands on miR-451 expression, we observed that only Tam had the ability to downregulate miR-451 and that downregulation occurred as early as 4 h, whereas the estrogen estradiol (E2), the SERM raloxifene (Ral) and the pure antiestrogen ICI182,780

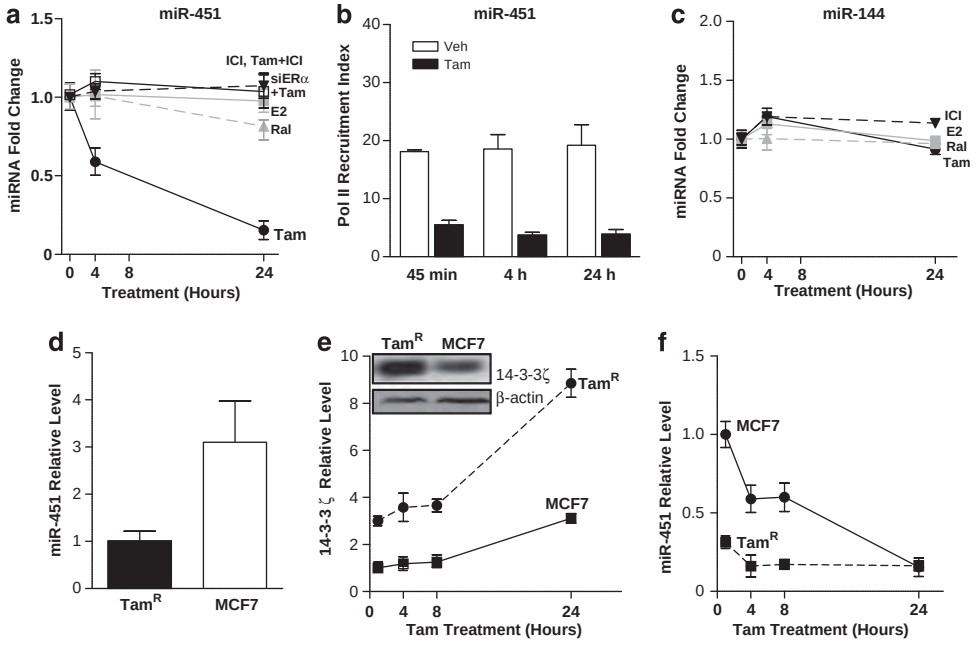

Figure 1 miR-451 is downregulated by tamoxifen in ER-positive breast cancer cells and its expression is inversely correlated with 14-3-3ζ in MCF7 and tamoxifen-resistant (Tam^R) cells. (**a**) Regulation of miR-451 in MCF7 cells by the estrogen receptor ligands Tamoxifen (Tam, 1 μM), Raloxifene (Ral, 1 μM), estradiol (E2, 10 nM) and ICI 182,780 (ICI Fulvestrant, 1 μM), or 0.1 μM Tam plus 3 μM ICI, after 0, 4 and 24 h of treatment. ERα was also depleted from cells by treatment with siRNA for ERα before Tam treatment for 0, 4 and 24 h. (**b**) RNA Polymerase II (Pol II) recruitment to the TSS of miR-451 in the presence of Veh or Tam for the times indicated. (**c**) Absence of regulation of the adjacent miR-144 by the four estrogen receptor ligands. (**d**) Levels of miR-451 in MCF7 and Tam^R breast cancer cells detected by quantitative PCR. (**e**, **f**) Change in 14-3-3ζ and miR-451 levels analyzed by quantitative RT–PCR in MCF7 and Tam^R cells after treatment with Tam. Values are fold expression compared with Veh control. Each experiment was performed in triplicate with three experimental replicates. The inset in panel **e** shows 14-3-3ζ detected by western blot.

(ICI, Fulvestrant) had no effect on miR-451. The downregulation of miR-451 by Tam was reversed by excess ICI, implying mediation of the Tam effect by ER. Likewise, depletion of ERα by *ca* 90% by cell treatment with siRNA targeting ERα (Chang *et al.,* 2008) eliminated the downregulation of miR-451 by tamoxifen (Figure 1a).

To further explore the regulation of miR-451 by tamoxifen, we analyzed the presence over time of RNA polymerase II (Pol II) at the transcription start site of miR-451 (transcription start site derived from the data of Welboren *et al*., 2009) in cells treated with control vehicle (Veh) or Tam^R. Interestingly, we found that Tam treatment markedly reduced the level of Pol II at the transcription start site of miR-451 (Figure 1b), consistent with the downregulation of miR-451 expression we observed upon treatment with tamoxifen.

The highly conserved miR-451 gene is located on chromosome 17, only 100 bp upstream of another miR, miR-144. We therefore examined if this nearby miR-144 was also regulated by Tam or other ER ligands. As shown in Figure 1c, we observed that Tam had no effect on the expression of miR-144, indicating the selectivity of tamoxifen for miR-451 regulation.

We next examined the levels of miR-451 in parental MCF7 and in tamoxifen-resistant MCF7 (Tam^R) cells and found that parental cells expressed three-times more miR-451 than did Tam^R cells (Figure 1d), whereas Tam^R cells had higher levels of 14-3-3ζ (Figure 1e), suggesting an inverse relationship between these two factors. Further, upon treatment of both MCF7 and Tam^R cells with tamoxifen, we observed a time-dependent decrease in miR-451 and an increase in 14-3-3ζ over time (Figures 1e and f). A similar inverse relationship between miR-451 and 14-3-3ζ was observed in other tamoxifen-resistant MCF7 cells selected in our laboratory (data not presented).

Overexpression or knockdown of miR-451 impacts 14-3-3ζ, cell proliferation and colony formation, apoptosis, and sensitivity to SERMs

To further investigate miR-451 regulation of 14-3-3ζ, we overexpressed miR-451 by transfecting pri-miRNA into MCF7 and Tam^R cells and monitored mRNA and protein levels of 14-3-3ζ by quantitative PCR and western blot. As shown in Figures 2a and b,

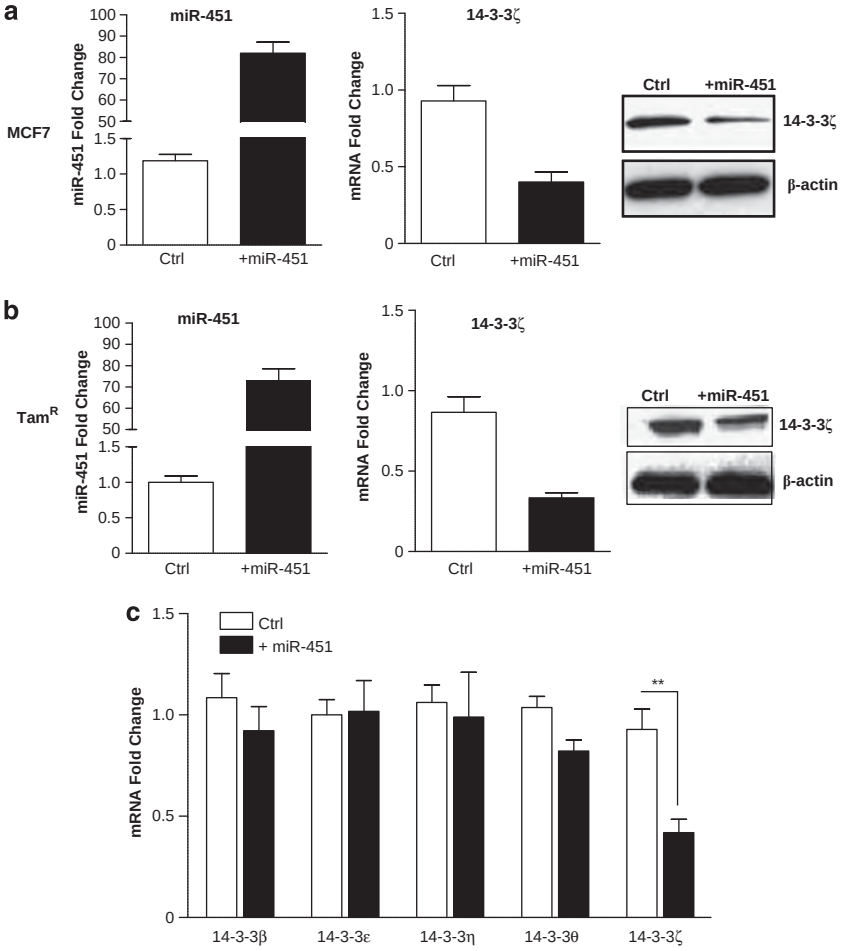

Figure 2 Regulation of 14-3-3ζ by miR-451 overexpression in MCF7 and Tam^R cells. (**a**, **b**) Overexpression of miR-451 resulted in the downregulation of 14-3-3ζ mRNA (quantified by RT–quantitative PCR) and protein (by western blot). Control (Ctrl) cells received vector only. Each experiment was performed in triplicate and mean ± s.d. is shown. (**c**) Expression of five 14-3-3 family members in MCF7 cells without and with miR-451 overexpression. $^{**}P<0.01$.

overexpression of miR-451 greatly reduced the level of 14-3-3ζ in both cell lines. We also examined the specificity of miR-451 action for targeting only 14-3-3ζ and not other 14-3-3 family members, and found that only 14-3-3ζ was significantly affected by expression of miR-451 (Figure 2c).

We next examined the impact of miR-451 status on proliferation and apoptosis of breast cancer cells resistant to Tam, by either overexpressing or knocking down miR-451. As shown in Figure 3a, Tam acted as an agonist in Tam^R cells, enhancing the proliferation of these cells over Veh control. Cells transfected with miR-451 showed greatly reduced proliferation and notably, this suppressive effect of miR-451 on proliferation was reversed by expression of 14-3-3ζ (Figure 3a). Also, knockdown of miR-451 (miR-451 KD) by treatment of cells with miR-451 antagomir, which suppressed endogenous miR-451 and increased 14-3-3ζ levels (Figure 3b), increased cell proliferation (Figure 3c). To determine the impact of 14-3-3ζ alone on cell viability, we downregulated its expression by siRNA and observed a significant decrease in basal and tamoxifen-stimulated cell proliferation (Figure 3d). Overexpression of miR-451, which reduced 14-3-3ζ without affecting ERα protein or mRNA levels (Figure 3f), resulted in suppression of the growth of Tam^R cells in the presence of the SERMs tamoxifen or Raloxifene, or Fulvestrant (ICI 182,780) (Figure 3e). After overexpression of miR-451, we also observed a marked reduction in anchorage-independent colony formation of Tam^R cells, that is, a significant decrease in number and size of colonies in the Veh and Tam-treated cells, suggesting a potential tumor suppressor-like activity of this miR (Figure 3g).

To understand the basis of the reduced cell viability and colony formation in cells overexpressing miR-451, we monitored 14-3-3ζ, CASP7 and cleaved CASP7 protein upon Tam treatment. As seen in Figure 4a, we observed greatly reduced 14-3-3ζ protein levels with miR-451 overexpression, as expected, and also elevated levels of CASP7 and cleaved CASP7. When 14-3-3ζ was re-expressed in cells overexpressing miR-451, the CASP7 and cleaved CASP7 levels were greatly decreased, becoming comparable to the low levels seen in control cells, indicating that miR-451, by altering intracellular14-3-3ζ, not only inhibits proliferation as shown in Figure 3, but also stimulates the apoptotic cascade. These changes were also reflected in the marked increase in proportion of cells in the sub-G1 phase of the cell cycle with miR-451 overexpression

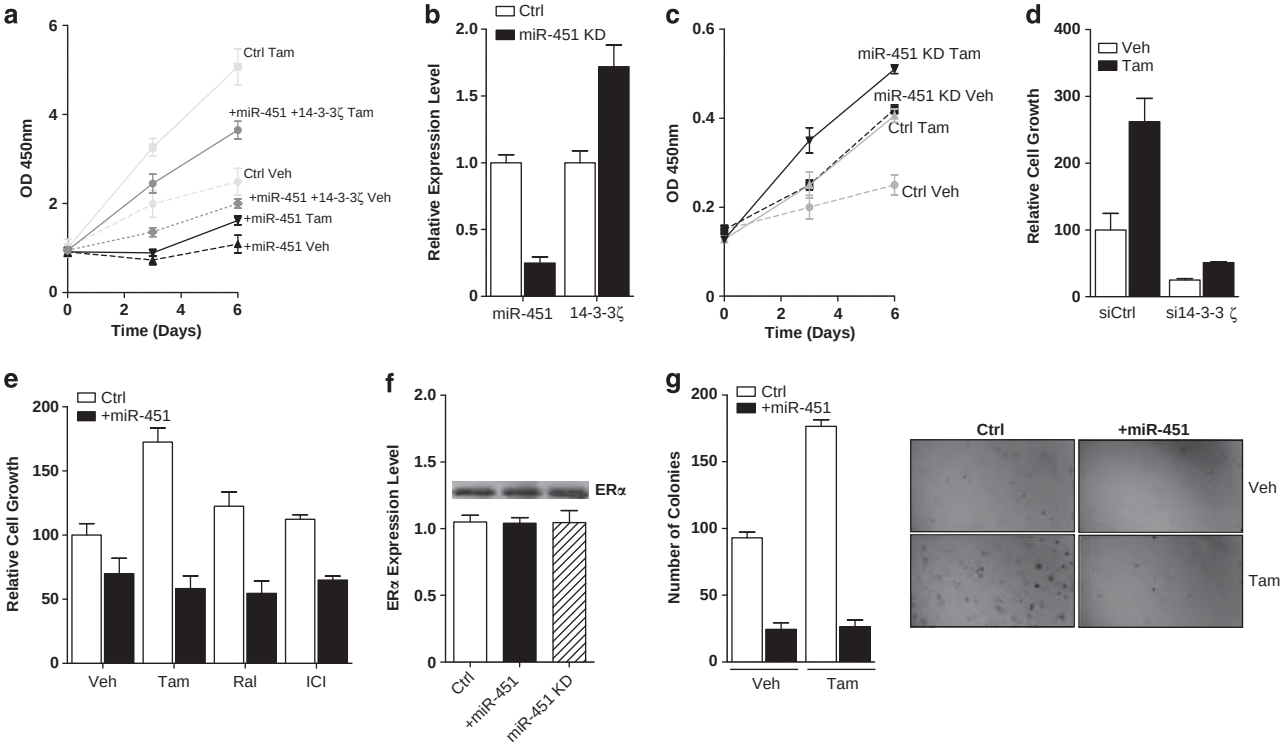

Figure 3 The cellular level of miR-451 affects growth and colony formation. (**a**) Growth of Tam^R cells in the presence of Tam or Veh was followed for up to 6 days after transfection of pri-miR-451 (+miR-451) or Ctrl vector (Ctrl) or pri-miR-451 and 14-3-3ζ (+miR-451 + 14-3-3ζ). The growth index was assessed at day 0, 3 and 6. (**b**) Effect of antagomiR-451transfection (denoted miR-451 KD) on miR-451 and 14-3-3ζ expression levels. (**c**) Effect of miR-451 KD on viability of Tam^R cells. Cell viability was assessed at day 0, 3 and 6 of Tam or Veh treatment. (**d**) After 14-3-3ζ knockdown by siRNA, growth of Tam^R cells in the presence of Tam or Veh was evaluated at day 6. (**e**) Sensitivity to SERMs was assessed after 72-h treatment with control Veh, or 1 μM Tam, Raloxifene or ICI in cells with control plasmid (Ctrl) or with miR-451 overexpression. (**f**) miR-451 overexpression or miR-451 downregulation has no impact on ERα mRNA or protein level. (**g**) Tam cells were assayed for soft agar colony formation after overexpression of miR-451 or control plasmid and exposure to Tam or Veh. Number of colonies and colony size were assessed and photography of colonies is also presented.

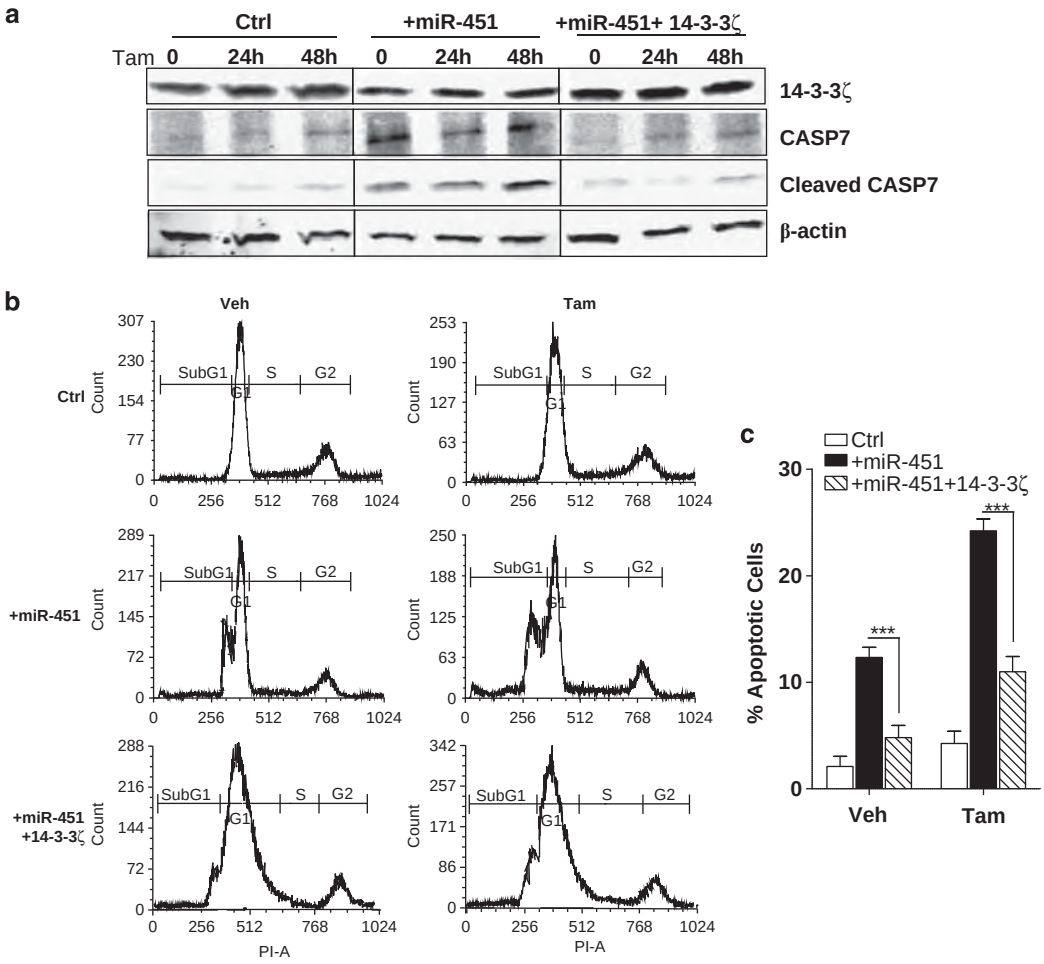

Figure 4 Increasing the expression of miR-451 reduces 14-3-3ζ and stimulates apoptosis, and the effects of miR-451 are reversed by expression of 14-3-3ζ. (**a**) Western blots of whole-cell extracts from vector, miR-451 alone, or miR-451 and 14-3-3ζ-transfected MCF7 cells treated with Tam for 0, 24 or 48 h and probed with antibodies against 14-3-3ζ and caspase-7 to detect intact and cleaved CASP7 products. β-Actin was used as loading control. (**b**) Control vector, miR-451, or miR-451 and 14-3-3ζ-expressing Tam^R cells were treated with 1 μM Tam or control Veh for 72 h, then fixed and stained with propidium iodide, and cell cycle distribution was monitored by flow cytometry. (**c**) The percent of apoptotic cells (sub-G1 peak) in control vector (Ctrl), miR-451, and miR-451 and 14-3-3ζ-expressing cells is presented. $***P<0.001$.

(Figure 4b). There also was a great increase in the percent of apoptotic cells, rising from 2 to 13% and from 5 to 24% in Veh- and Tam-treated cells, respectively (Figure 4b). The proportion of cells in the sub-G1 phase of the cell cycle and the percent of apoptotic cells were significantly reduced when 14-3-3ζ was re-expressed in cells overexpressing miR-451 (Figures 4b and c).

Elevated levels of miR-451 reduce the activation of growth factor receptor tyrosine kinases and protein kinases

As endocrine resistance is often associated with the upregulation of growth factor receptor and protein kinase signaling, we evaluated the effects of 14-3-3ζ and miR-451 on the activation of some growth factor receptors and kinases. We observed a significant increase in phosphorylation of HER2, EGFR, AKT and MAPK upon 14-3-3ζ overexpression (Figure 5a). We also examined the impact of miR-451 on these receptors and downstream signaling pathways, and observed that levels of phosphoHER2 and phosphoEGFR were greatly reduced in cells overexpressing miR-451, and pMAPK was also decreased, although only a slight reduction was observed in pAKT (Figure 5b).

Selective action of miR-451 on 14-3-3ζ controls cell proliferation

To further delineate the importance of 14-3-3ζ in the actions of miR-451, we used a selective 14-3-3ζ target protector to examine the specificity of miR-451 action. Of the 14 targets for miR-451 predicted by the TargetScan algorithm, we found 8 to be expressed in MCF7 cells. Knockdown of endogenous miR-451 increased only the level of 14-3-3ζ (Figure 6a). However, with high overexpression of miR-451, 14-3-3ζ was

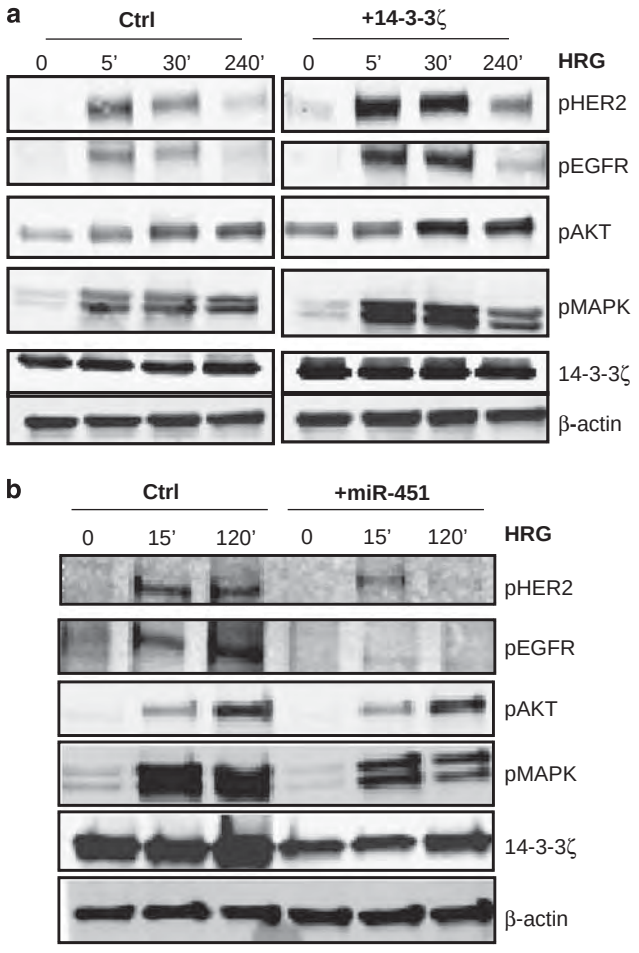

Figure 5 miR-451 and 14-3-3ζ affect the activation of receptor tyrosine kinases and protein kinases. Immunoblotting for pHer2, pEGFR, pAKT, pMAPK and 14-3-3ζ in Tam^R cells treated with heregulin (HRG) for the minutes indicated after cells were transfected with (**a**) control or 14-3-3ζ-expressing polylysine-coated adenovirus or (**b**) control or pre-miR-451 vector to overexpress miR-451. β-Actin was used as a loading control.

downregulated along with two other factors, *O*-GlcNAc transferase (OGT) and cyclin-dependent N-kinase 2D (CDKN2D) (Figure 6a).

As shown in Figure 6b, in control cells, tamoxifen only upregulated 14-3-3ζ, and had no effect on OGT or CDKN2D. These observations suggest that OGT and CDKN2D are less sensitive to miR-451 and, unlike 14-3-3ζ, are not suppressed by endogenous levels of this miR. To examine whether 14-3-3ζ was primarily responsible for the impact of miR-451 on cellular behavior, we utilized an RNA-binding antisense oligonucleotide specific for the interaction between miR-451 and the 3′-UTR of 14-3-3ζ (target protector), so as to disrupt only this interaction. We monitored the levels of 14-3-3ζ, OGT and CDKN2D in cells overexpressing miR-451 or 14-3-3ζ protector alone, or both combined (Figure 6b). Overexpression of miR-451 reduced the expression of all three, but the addition of the 14-3-3ζ protector in miR-451-overexpressing cells restored the basal level and tamoxifen response of only 14-3-3ζ, reversing the effect of miR-451 overexpression. By contrast, there was no effect of the protector on OGT and CDKN2D with miR-451 overexpression. In cells exposed to 14-3-3ζ protector alone, there was an increase in the basal (Veh) level of 14-3-3ζ but no effect on OGT or CDKN2D, as would be expected from reduction in the effect of endogenous miR-451 on 14-3-3ζ.

We then examined the effect of these perturbations on the growth of Tam^R cells (Figure 6c). As shown previously in Figure 3, miR-451 knockdown increased 14-3-3ζ and cell proliferation, whereas miR-451 overexpression suppressed both basal and tamoxifen-stimulated proliferation, and these were restored to the levels in control (Ctrl) cells by co-presence of the 14-3-3ζ protector (Figure 6c). The protector alone raised the proliferation rate of Veh-treated cells, consistent with its effect on the endogenous 14-3-3ζ level, shown in Figure 6b, left panel. Collectively, these results support the hypothesis that the effects of both up- and downregulation of miR-451 on cell proliferation and response to tamoxifen are mediated principally by miR-451 regulation of 14-3-3ζ levels. Our overall findings, schematically depicted in the model in Figure 7, show that tamoxifen decreases endogenous miR-451, thereby increasing the level of 14-3-3ζ. 14-3-3ζ promotes breast cancer cell proliferation, survival and receptor tyrosine kinase (EGFR, HER2) activation, and protein kinase signaling while suppressing apoptosis, all of which support the progression to endocrine resistance.

Discussion

The development of resistance to endocrine therapy is a severe limitation in the treatment of hormone-receptor-positive breast tumors. In this study, we provide evidence for a novel mechanism by which tamoxifen controls 14-3-3ζ levels through its regulation of the miR, miR-451. It is becoming increasingly clear that miRNAs have a profound impact on many pathological and physiological processes, including proliferation, differentiation and apoptosis (Bartel, 2004; Harfe, 2005), by dampening the expression of target genes and thereby affording finely tuned cellular regulation. Lowered mRNA levels appear to be the predominant mode of miR regulation, although decreased translational efficiency often contributes to reduced protein output as well (Guo *et al*., 2010).

Our previous studies described the upregulation of 14-3-3ζ by tamoxifen and revealed that high expression of 14-3-3ζ in primary breast cancers was associated with a poor clinical outcome on tamoxifen (Frasor *et al*., 2006). 14-3-3ζ, which is harbored in a region of frequent genomic gain (8q23), is overexpressed in greater than 40% of breast tumors (Neal *et al*., 2009) and is overexpressed also in other types of aggressive solid tumors, such as lung cancer (Li *et al*., 2008). Thus, 14-3-3ζ has the properties of an oncogene; yet

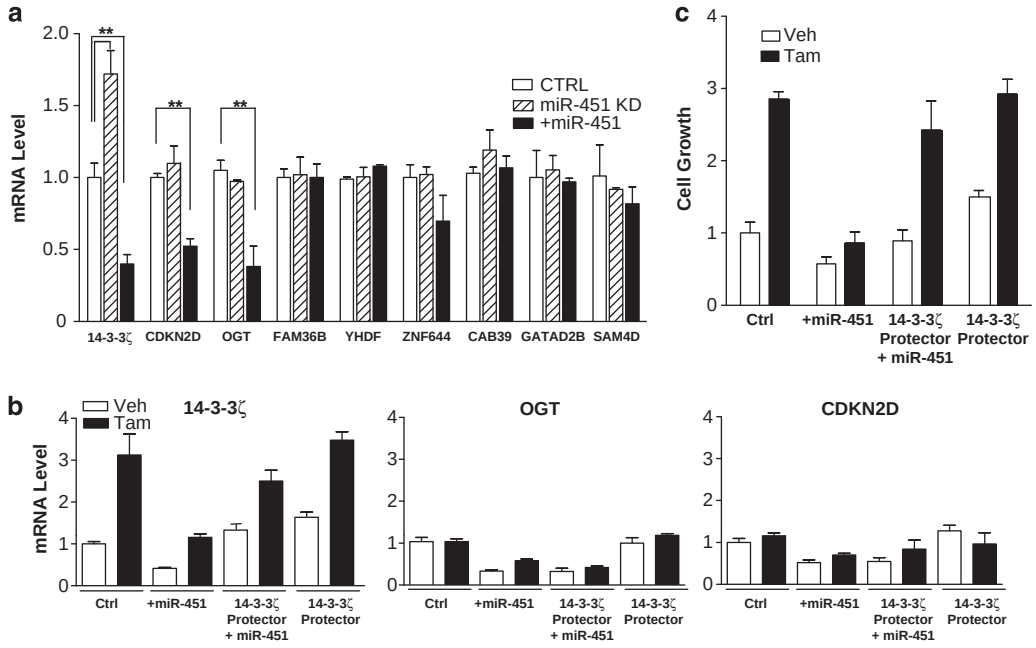

Figure 6 Effects of miR-451 knockdown or overexpression on potential target genes. (**a**) Expression levels of predicted target genes of miR-451, after knockdown of endogenous miR-451 or overexpression of miR-451 (**$P<0.01$). (**b**) Quantitative PCR detection of expression levels of 14-3-3ζ, OGT or CDKN2D in Veh- or 1 μM Tam-treated cells, after control vector (Ctrl) or after miR-451 overexpression and/or 14-3-3ζ target protector exposure. (**c**) Growth of Tam^R cells, with veh or 1 μM Tam treatment, after control vector (Ctrl) or after miR-451 overexpression and/or 14-3-3ζ target protector exposure.

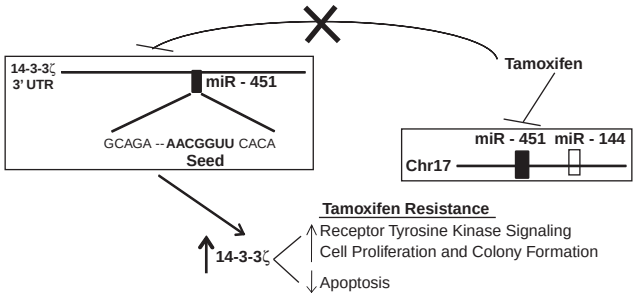

Figure 7 Schematic representation of the effect of tamoxifen on miR-451 and 14-3-3ζ regulation and their impact on breast cancer cell phenotypic properties leading to tamoxifen resistance. Tamoxifen downregulates miR-451, resulting in the upregulation of 14-3-3ζ, with consequent increased receptor tyrosine kinase signaling, increased cell proliferation and colony formation, and reduced apoptosis, thereby leading to tamoxifen resistance.

surprisingly, its regulation in breast cancer has been largely unknown.

It is of note that the regulation of 14-3-3ζ and miR-451 is selective for tamoxifen and is not brought about by other ER ligands tested, including the estrogen estradiol or the antiestrogens raloxifene and ICI 182,780, highlighting the remarkable ability of distinct ER–ligand complexes to selectively affect the transcription of specific genes (Katzenellenbogen *et al*., 1996; Katzenellenbogen and Katzenellenbogen, 2002; Shang and Brown, 2002; Frasor *et al*., 2004, 2006). In all cases examined, we observed an inverse relationship between miR-451 and 14-3-3ζ, implying the importance of this miRNA in the regulation of 14-3-3ζ. This regulation of miR-451 by tamoxifen was also highly selective, as tamoxifen did not affect the nearby miR-144. The differential regulation of miR-451 and 14-3-3ζ by tamoxifen vs raloxifene is reminiscent of other differences also observed by us previously when genome-wide gene expression comparison analyses of these two SERMs were performed (Frasor *et al*., 2004). These differential effects of the two SERMs in regulation of miR-451 and 14-3-3ζ may underlie some differences in their activities observed in the STAR (Study of Tamoxifen and Raloxifene) breast cancer prevention trial (Dunn and Ford, 2001; Vogel *et al*., 2006, 2010).

Previous reports have noted that miR-451 is very highly conserved among vertebrates, and that its maturation is independent of Dicer (Yang *et al*., 2010). Moreover, miR-451 has been shown recently to have a crucial role in erythrocyte differentiation by targeting 14-3-3ζ (Masaki *et al*., 2007; Dore *et al*., 2008; Patrick *et al*., 2010; Yu *et al*., 2010). Expression of miR-451 has also been detected in normal gastric mucosa and found to be decreased in gastric cancer (Bandres *et al*., 2009). These findings imply that miR-451 is required for the development and maintenance of normal tissues (Williams *et al*., 2007) and may be downregulated during the progression to cancer. Of note, we found that our less-aggressive cell line (MCF7) showed higher expression of miR-451 compared with the more-aggressive tamoxifen-resistant cell line. We have also observed upregulation of 14-3-3ζ and downregulation of miR-451 by tamoxifen in ER-positive, HER2-positive BT474 cells (data not presented). Therefore, the loss of miR-451 induced by Tam treatment appears to be an

underlying mechanism by which ER-positive breast cancer cells become resistant to tamoxifen therapy by upregulation of 14-3-3ζ. As supporting evidence for this, we show that reduction of miR-451 by tamoxifen treatment or knockdown of miR-451 by a specific antagomir increased 14-3-3ζ and cell survival, whereas increasing miR-451 could resensitize Tam^R cells to SERMs, as observed by the decrease in cell viability and suppression of anchorage-independent growth that correlated with increased apoptosis and reduced EGFR, HER2 and MAPK signaling in tamoxifen-treated miR-451-overexpressing cells. This sensitization of tamoxifen-resistant cells to the growth-inhibitory effects of Tam and other antiestrogens by increased expression of miR-451 suggests that restoration of expression of this miRNA in endocrine therapy-resistant cancer cells might have important implications for effective breast cancer treatment.

In summary, we document miR-451 to be a potent suppressor of the progression to tamoxifen resistance through its ability to target 14-3-3ζ, a key proliferative and antiapoptotic factor in breast cancer. Modulation of miR-451 status impacted growth, apoptosis, receptor tyrosine kinase activity and sensitivity to SERMs. We identify miR-451 as a target of the ER–tamoxifen complex and show an inverse correlation between cellular miR-451 and 14-3-3ζ levels. Collectively, these data imply that increasing the level of miR-451 may have beneficial therapeutic effects in the large proportion of breast cancers expressing high levels of 14-3-3ζ. Progress in miR-directed therapeutic approaches (Lowery *et al*., 2008; Shan *et al*., 2008) offers hope that such strategies might prove useful in reversing endocrine resistance and reducing breast cancer recurrence.

Materials and methods

Cell cultures, constructs and cell transfections
MCF7 cells, obtained from the American Type Culture Collection (Manassas, VA, USA) and tamoxifen-resistant MCF7 cells (Tam^R cells) described previously (Herman and Katzenellenbogen, 1996), were cultured in MEM (Sigma-Aldrich Corp., St Louis, MO, USA), supplemented with 5% calf serum (HyClone, Logan, UT, USA), 100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and 25 μg/ml gentamicin (Invitrogen). Four days before control Veh or ligand treatments, cells were seeded in phenol red-free MEM containing 5% charcoal-dextran-treated calf serum. The medium was changed on day 2 and 4 of culture before treatments. Pri-miRNA-451 was purchased from Origene (Rockville, MD, USA) and antagomiR-451, antagomiR-144 and negative control antagomiRs were purchased from Applied Biosystems (Carlsbad, CA, USA). A 40-nt single-stranded RNA-binding antisense oligonucleotide specific for the interaction between miR-451 and the 3′-UTR of 14-3-3ζ (target protector) was purchased from Qiagen (Valencia, CA, USA). siRNA targeting ERα was as described previously (Chang *et al*., 2008). Ectopic overexpression of 14-3-3ζ used lysine-coated adenovirus or control adenovirus (Allgood *et al*., 1997). Cell transfections were performed with Fugene 6 (Roche) or Dharmafect (Dharmacon, Lafayette, CO, USA).

RT–PCR and quantitative PCR
Total RNA was isolated from cells using TRIzol (Invitrogen), RNA samples were reverse transcribed by SuperScript II reverse transcriptase (Invitrogen) and real-time PCR was carried out on the ABI Prism 7900HT using SYBR Green PCR Master Mix (Applied Biosystems) as described previously (Frasor *et al*., 2006).

Western blot analysis
Whole-cell extracts were prepared using 1X RIPA lysis buffer (Upstate/Chemicon, Temecula, CA, USA) supplemented with 1 × complete protease inhibitor (Roche, Basel, Switzerland). Proteins were separated on 15% SDS–PAGE gels and transferred to nitrocellulose membranes. Western blotting used antibodies against 14-3-3ζ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Sigma-Aldrich Corp), pEGFR, pHER2, pMAPK and pAKT (Cell Signaling, Danvers, MA, USA).

Cell proliferation assays
WST-1 assay (Roche, Basel, Switzerland) was used to quantify cell viability. Absorbance was measured at 450 nm using a BioRad 680 Microplate Reader (BioRad, Hercules, CA, USA), and all assays were performed in triplicate.

Apoptosis assays
Apoptosis was measured based on DNA content and analyzed by flow cytometry using BD-FACS Canto. Cells were fixed in 70% ethanol, stained for 30 min with 20 μg/ml propidium iodide (PI, Molecular Probe, Carlsbad, CA, USA) in Triton-X (Sigma) in the presence of DNAse-free RNAse A (Sigma), and PI staining was measured as previously reported (Riccardi and Nicoletti, 2006).

Soft-agar colony formation assays
A 1.5-ml base layer of agar (0.5% agar in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) with 5% charcoal stripped-FCS) was allowed to solidify in a six-well flat-bottomed plate before the addition of 1.5 ml of cell suspensions containing 4000 cells in 0.35% agar in phenol red-free DMEM with 5% charcoal-stripped FCS. The cell-containing layer was then solidified at 4 °C for 20 min. Colonies were allowed to grow for 13 days at 37 °C before imaging.

Abbreviations

ER, estrogen receptor; ICI, ICI 182,780, fulvestrant; miR, microRNA; Ral, raloxifene; SERM, selective estrogen receptor modulator; Tam, tamoxifen.

Conflict of interest

The authors declare no conflict of interest.

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Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

Reversal of endocrine resistance in breast cancer: interrelationships among 14-3-3ζ, FOXM1, and a gene signature associated with mitosis

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Abstract

Introduction: Despite the benefits of estrogen receptor (ER)-targeted endocrine therapies in breast cancer, many tumors develop resistance. 14-3-3 ζ/YWHAZ, a member of the 14-3-3 family of conserved proteins, is over-expressed in several types of cancer, and our previous work showed that high expression of 14-3-3ζ in ER-positive breast cancers was associated with a poor clinical outcome for women on tamoxifen. Therefore, we now probe the role of 14-3-3ζ in endocrine resistance, and we examine the functional dimensions and molecular basis that underlie 14-3-3ζ activities.

Methods: From analyses of four independent breast cancer microarray datasets from nearly 400 women, we characterized a gene signature that correlated strongly with high expression of 14-3-3ζ in breast tumors and examined its association with breast cancer molecular subtypes and clinical-pathological features. We investigated the effects of altering 14-3-3ζ levels in ER-positive, endocrine sensitive and resistant breast cancer cells on the regulation of 14-3-3ζ signature genes, and on cellular signaling pathways and cell phenotypic properties.

Results: The gene signature associated with high 14-3-3ζ levels in breast tumors encompassed many with functions in mitosis and cytokinesis, including aurora kinase-B, polo-like kinase-1, CDC25B, and BIRC5/survivin. The gene signature correlated with early recurrence and risk of metastasis, and was found predominantly in luminal B breast cancers, the more aggressive ER-positive molecular subtype. The expression of the signature genes was significantly decreased or increased upon reduction or overexpression of 14-3-3ζ in ER-positive breast cancer cells, indicating their coregulation. 14-3-3ζ also played a critical role in the regulation of FOXM1, with 14-3-3ζ acting upstream of FOXM1 to regulate cell division-signature genes. Depletion of 14-3-3ζ markedly increased apoptosis, reduced proliferation and receptor tyrosine kinase (HER2 and EGFR) signaling, and, importantly, reversed endocrine resistance.

Conclusions: This study reveals that 14-3-3ζ is a key predictive marker for risk of failure on endocrine therapy and serves a pivotal role impacting growth factor signaling, and promoting cell survival and resistance to endocrine therapies. Targeting 14-3-3ζ and its coregulated proteins, such as FOXM1, should prove valuable in restoring endocrine sensitivity and reducing risk of breast cancer recurrence.

Keywords: estrogen receptor, antiestrogens, endocrine resistance, gene expression, 14-3-3ζ?ζ?

Introduction

Approximately 70% of breast cancers are positive for estrogen receptor (ER) α at diagnosis, and these patients often benefit from endocrine therapies that target ER, because the proliferative drive of these tumors and many of their phenotypic properties result from estrogens acting through the ER [1]. ER is a master regulator of gene expression in breast cancer, upregulating survival and proliferation-promoting factors and downregulating proapoptotic and tumor suppressing factors [1-4]. Endocrine therapies in breast cancer, when effective, are desirable because they are generally well tolerated and avoid the morbidity associated with radiation and chemotherapies.

All forms of endocrine therapies, including ER antagonists such as the selective estrogen receptor modulators

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(SERMs) tamoxifen and raloxifene, and the selective ER downregulator (SERD) fulvestrant, function by interrupting estrogen signaling through the ER. These therapies targeting the ER have profoundly impacted breast cancer treatment and improved patient survival [5]. The benefit of endocrine therapies, however, is limited by the development of resistance, a process that appears to result from upregulation of growth factor and protein kinase signaling pathways that provide an alternate mechanism in support of tumor cell proliferation and survival [6-9]. Hence, there is great interest in identifying and targeting, by inhibition or downregulation, key factors that mediate endocrine resistance.

We previously identified 14-3-3ζ, also known as YWHAZ, from gene expression profiling on a cohort of ER-positive breast tumor samples and found that women whose tumors had high levels of 14-3-3ζ showed a poor clinical outcome on tamoxifen [10]. However, the molecular mechanisms underlying this poor clinical response on endocrine therapy still remain unknown. 14-3-3ζ is a member of a highly conserved family of 14-3-3 proteins, and it functions as a scaffold or platform that regulates the activity and stability of interacting proteins by binding to their phosphoserine and phosphothreonine motifs [11-15]. Therefore, we have undertaken studies to probe the functional dimensions of 14-3-3ζ activity and its mechanistic basis in which we: characterize a gene signature associated with overexpression of 14-3-3ζ in breast tumors; determine the association of 14-3-3ζ with the molecular subtypes and clinical pathological features of breast cancers; identify the gene regulations, cellular pathways, and cell phenotypic properties modulated by 14-3-3ζ status; and examine the role of 14-3-3ζ in breast cancer endocrine resistance. Our studies reveal that 14-3-3ζ is a key survival factor integrating proliferative inputs from multiple cellular pathways, and that downregulation of 14-3-3ζ can restore endocrine sensitivity in resistant breast cancer cells. The findings suggest that targeting 14-3-3ζ or the proteins it regulates could be a useful approach for enhancing and prolonging the effectiveness of endocrine therapies.

Materials and methods

Analysis of microarray datasets and identification of a 14-3-3ζ gene signature

Microarray gene expression analysis and data processing were from four independent clinical studies encompassing 390 ER-positive primary breast tumors [10,16-18]. From the Frasor *et al*. dataset [10], we included the 67 ER-positive tumors from patients who subsequently underwent endocrine therapy with tamoxifen and microarrays were analyzed as described therein. From the van't Veer et al. dataset, we included 47 ER-positive breast tumors and associated expression data, and clinical data were obtained from Rosetta Inpharmatics (Kirkland, WA, USA) [17]. Downloaded log base 2 data were transformed to linear values and uploaded to GeneSpring GX 7.3 (Agilent Technologies, Santa Clara, CA, USA) From the Wang *et al*. dataset [18], we included 209 ER-positive breast tumors, and gene expression and clinical data were obtained from GEO (Series GSE2034). The downloaded data were transformed into GeneSpring GX 7.3 and chips and genes were median normalized and median polished. Log base 2 data from 67 ER-positive primary breast tumors from the Sorlie *et al*. cohort [16] were downloaded from GEO (Series 4335), uploaded to GeneSpring GX 7.3 and then chips and genes were median normalized. Frasor *et al*. [10] and Wang *et al*. [18] used the Hu133A-Affymetrix microarray platform; van't Veer *et al*. [17] used Hu25K-Agilent arrays; and Sorlie *et al*. [16] used cDNA Stanford arrays containing 8,102 genes. For the Sorlie *et al*. dataset, all the patients were treated with either doxorubicin or 5-fluorouracil and mitomycin C but no information on hormonal or other neo-adjuvant treatment was available. For Wang *et al*. and van't Veer *et al*., no treatments were publicly available or could be associated with any samples.

Hierarchical clustering of data was performed and displayed using Eisen Cluster and TreeView software for analysis and visualization. Based on 14-3-3ζ microarray expression levels, breast cancer samples [10] were divided into high (≥1.8 log2) and low (< 1.8 log2) 14-3-3ζ expression groups and a two-class statistical analysis of microarrays (SAM) was conducted [19]. Genes with FDR (false discovery rate) of 0.01 or less and with a fold change of three or more were included in the gene signature. The prediction analysis of microarrays method [20] was used as a cross-validation of the 14-3-3ζ signature.

Survival analysis

Patients were divided into high and low 14-3-3ζ gene signature expression groups and Kaplan-Meier curves were computed by the Cox-Mantel log-rank test in WinStat for Microsoft Excel R. Fitch, Germany).

Cell cultures and generation of stable cell lines

MCF7 cells, from the American Type Culture Collection (Manassas, VA, USA), and tamoxifen-resistant MCF-7 cells [21] were grown and treated as described [2,10]. Cells with stable knockdown of 14-3-3ζ (KD cells) were generated by transfection of pRNATin 5.1 (Ambion Austin, TX, USA) containing shRNA (TCTTGAGGTGGCCAATATTTC) targeting the 3' UTR. Cells were selected in the presence of hygromycin B (100 μg/ml). Some transfections utilized an adenovirus-mediated method [22].

Western blot analysis

Whole-cell extracts were prepared using 1X RIPA Lysis buffer (Upstate/Chemicon Billerica, MA, USA) supplemented with 1X complete protease inhibitor (Roche, Basel, Switzerland). Western blotting used antibodies against 14-3-3ζ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Sigma-Aldrich, St Louis, MO, USA), phosphoepidermal growth factor receptor (EGFR), phospho- human epidermal growth factor receptor 2 (HER2), phospho- mitogen activated protein kinase (MAPK) and phospho-AKT/PKB (protein Kinase B) (Cell Signaling, Danvers, MA, USA).

RT-PCR and quantitative PCR

Total RNA was isolated from cells using TRIzol, reverse transcribed by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and real-time PCR performed on the ABI Prism 7900HT using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) [10,23].

Cell proliferation, colony formation and apoptosis assays

The WST-1 assay was used to quantify cell viability (Roche, Basel, Switzerland) and absorbance was measured at 450 nm using a BioRad 680 Microplate Reader (BioRad, Hercules, CA, USA). All assays were performed in triplicate. For the colony formation assay, a 1.5 mL base layer of agar (0.5% agar in phenol red-free DMEM with 5% charcoal stripped-fetal calf serum) was allowed to solidify in a six-well flat-bottomed plate before the addition of 1.5 mL of cell suspensions containing 4,000 cells in 0.35% agar in phenol red-free DMEM with 5% charcoal stripped-FCS. The cell-containing layer was then solidified at 4°C for 20 minutes. Colonies were allowed to grow for 15 days at 37°C with 5% CO2 before imaging and counting. Apoptosis was monitored based on DNA content by flow cytometry using BD-FACS Canto. Cells were fixed in 70% ethanol, stained for 30 minutes with 20 ug/ml propidium iodide (PI, Molecular Probe, Carlsbad, CA, USA) in Triton-X (Sigma, St Louis, MO, USA) in presence of DNAse-free RNAse A, and PI staining was measured [24].

Results

A gene signature and molecular phenotype in primary breast tumors associated with overexpression of 14-3-3ζ

We previously reported that *trans*-hydroxytamoxifen specifically regulated the expression of a set of approximately 70 genes in ER-positive breast cancer cells. Of these, high 14-3-3ζ was associated with a poor clinical outcome for women on tamoxifen therapy [10]. To elucidate the role that 14-3-3ζ plays in engendering this poor clinical outcome, we sought to identify genes significantly associated with high level expression of 14-3-3ζ and to relate these to breast cancer phenotype and gain mechanistic insights into the functions of 14-3-3ζ. For this, we classified samples from our previously described cohort of 67 ER-positive primary breast tumors from women treated with tamoxifen [10] into two groups based on high or low 14-3-3ζ expression and employed two-class SAM analysis and retrieved 29 genes with an FDR of 0.01 or less and a fold change of three or more (Table 1). Using the DAVID database [25] to classify our signature gene list based on Gene Ontology terms, we found that 46% of the genes in this signature were significantly enriched in the "cell cycle"

Table 1 List of genes in the 14-3-3ζ gene signature, based on SAM analysis

Symbol	Name	UGRepAccession
AURKB	Aurora kinase B	CD049340
BIRC5	Effector cell peptidase receptor 1	NM_001012271
BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog	AF053305
CDC20	Cell division cycle 20 homolog	BG256659
CDC25B	Cell division cycle 25 homolog B	NM_021873
CDCA8	Cell division cycle associated 8	BC000703
CENPA	Centromere protein A	BM911202
CEP55	Centrosomal protein 55kDa	NM_018131
CKS2	CDC28 protein kinase regulatory subunit 2	BQ898943
CYC1	Cytochrome c-1	BF569085
DGAT1	Diacylglycerol O-acyltransferase homolog 1	XM_001719374
EXOSC4	Exosome component 4	BM911415
FAM82B	Family with sequence similarity 82, member B	NM_016033
GPR172A	G protein-coupled receptor 172A	CR625605
HMMR	Hyaluronan-mediated motility receptor (RHAMM)	AF032862
HSPB8	Heat shock 22kDa protein 8	NM_014365
KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	BC067848
NDRG1	N-myc downstream regulated gene 1	NM_006096.3
PCSK1N	Proprotein convertase subtilisin/kexin type 1 inhibitor	BM805628
PLK1	Polo-like kinase 1	AB209179
RECQL4	RecQ protein-like 4	BC020496
SLC16A3	Solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	NM_001042422
SLC39A4	Solute carrier family 39 (zinc transporter), member 4	AK056900
SQLE	Squalene epoxidase	NM_003129
TPX2	TPX2, microtubule-associated, homolog	NM_012112
TRIP13	Thyroid hormone receptor interactor 13	NM_004237
UBE2C	Ubiquitin-conjugating enzyme E2C	BC032677
UBE2S	Ubiquitin-conjugating enzyme E2S	BM479313
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	BC051814

category ($P \leq 0.0001$). Among these were BUB1 (budding uninhibited by benzimidazoles 1 homolog), BIRC5/Survivin, CDCA8 (cell division cycle-associated protein 8), AURKB (aurora kinase B), CDC25B (cell division cycle 25 homolog B), and PLK1 (polo-like kinase 1), genes involved in mitosis and cytokinesis that tightly clustered with 14-3-3ζ (Figure 1a).

To analyze further how these genes might help explain the molecular phenotype of tumors overexpressing 14-3-3ζ, we performed unsupervised hierarchical clustering analysis and identified two main groups of patients based on 14-3-3ζ signature gene expression. When Kaplan-Meier analysis was performed using relapse as an endpoint, patients with breast tumors having high expression of these genes (High Signature Expression) showed a significantly poorer outcome (Figure 1b).

To further assess the relevance and applicability of this 14-3-3ζ signature, we selected ER-positive tumors from three other independent breast tumor microarray datasets with clinical information available. For all these three datasets no information on hormonal treatment

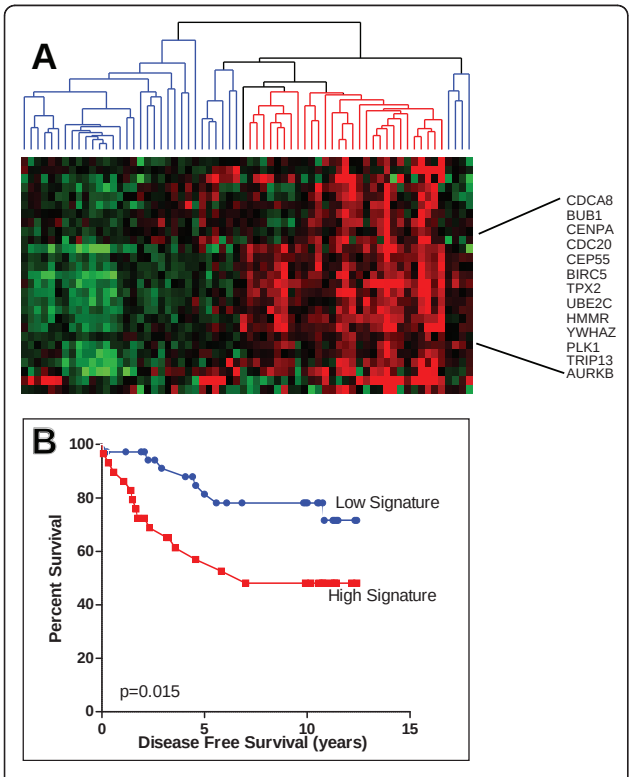


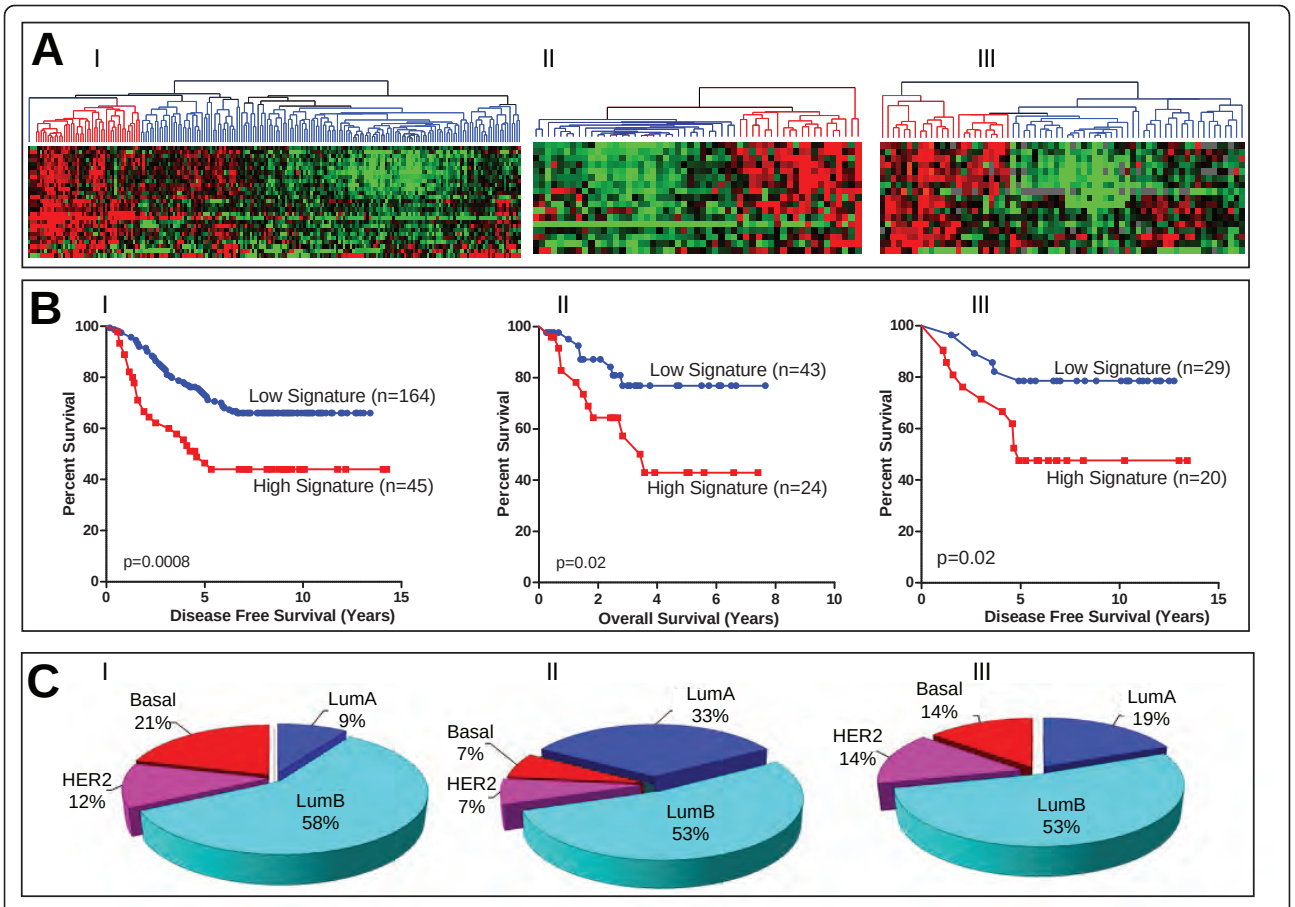
Figure 1 Identification of a 14-3-3ζ gene signature. **(a)** 29 genes associated with high levels of 14-3-3ζ were identified and clustered based on their expression profile. Hierarchical clustering identified a subgroup of patients (red) characterized by elevated levels of mitosis and cytokinesis related genes. **(b)** Kaplan-Meier curves for the red and blue clusters of the hierarchical diagram in panel (a) distinguished between good (blue, signature low expression) and poor prognosis (red, signature high expression) patients ($P = 0.015$).

was reported. Figure 2a shows the heat maps and dendrograms for expression of the 14-3-3ζ signature genes from these three studies (panel I Wang *et al.*, panel II van't Veer *et al.*, panel III Sorlie *et al.*). The red grouping in the dendrogram represents breast tumors with high expression of the signature genes. The Wang *et al.* dataset [26] includes data from 209 patients, and used the same Hu133A-Affymetrix microarray platform used by Frasor *et al.* [10]. All 29 genes from the 14-3-3ζ signature were retrieved and used for data mining. Unsupervised clustering analysis identified the red group (Signature High, $P = 0.001$ Kaplan-Meier, Figure 2b) Panel I as a poor prognosis group driven by high expression of the signature genes.

In a similar fashion, we analyzed the ER-positive breast tumors ($n = 49$) included in the van't Veer dataset [17]. Given the different microarray platform used (Hu25K-Agilent), a reduced number of genes were retrieved, 17 out of the 29 genes in the 14-3-3ζ gene signature. The signature genes not retrieved by our analysis were not present on those arrays. However, the subset of patients characterized by high expression of the 14-3-3ζ signature showed a significantly earlier relapse (Figure 2b, Panel II). We also examined the dataset of Sorlie *et al.* [16,27], which used cDNA Stanford arrays containing 8,102 genes. Expression data for 19 genes of the gene signature were recovered and used for the analysis. (The signature genes not recovered were not present on these arrays.) The findings confirmed once again that overexpression of the 14-3-3ζ signature was significantly associated with a poorer disease-free survival (Figure 2b, Panel III).

Breast cancer subtypes and the 14-3-3ζ gene signature

We next examined the distribution of the five major breast cancer molecular subtypes in the set of patients that showed high expression of the 14-3-3ζ gene signature and a poor clinical outcome in the different clinical studies by using a centroid-mediated clustering algorithm. All datasets showed enrichment for luminal-B subtypes in tumors with elevated expression of the 14-3-3ζ signature genes, ranging from 53 to 58% of all tumors (Figure 2c). In addition, 7 to 21% of total ER-positive breast cancers showing high expression of the 14-3-3ζ gene signature were represented by the basal breast cancer subtype. For comparison we also classified tumors characterized by low expression of the 14-3-3ζ gene signature, and found that luminal A was the most abundantly represented molecular subtype in the different datasets (data not shown). When correlated with clinical-pathological features, high level expression of the 14-3-3ζ gene signature was significantly associated with tumor grade, with the luminal-B vs. luminal-A breast cancer subtype, and with metastasis (Figure 2d).



D

14-3-3ζ Signature Dataset	Age	Size	Grade III vs I	PR+	Lymph Node+	Lum B vs Lum A	Metastasis
Frasor *et al.*	***0.04***	**0.021**	***0.001***	0.07	0.433	***0.001***	***0.008***
Sorlie *et al.*	NA	0.383	***0.001***	NA	0.430	***0.001***	0.384
van't Veer *et al.*	***0.010***	***0.002***	***0.020***	0.180	***0.040***	***0.001***	***0.012***
Wang *et al.*	NA	NA	NA	NA	NA	***0.001***	***0.019***

Figure 2 Association of signature gene expression with prognosis, breast cancer molecular subtypes, and clinical-pathological features. **(a)** Heat maps and dendrograms for Wang *et al.* (panel I), van't Veer *et al.* (panel II), and Sorlie *et al.* (panel III) datasets and expression of the 14-3-3ζ signature genes. From left to right, I) analysis of Wang *et al.* dataset comprising 209 estrogen receptor (ER)-positive breast tumors, II) analysis of 49 ER-positive tumors from the van't Veer *et al.* study and III) analysis of 67 ER-positive tumors from the Sorlie *et al.* study. **(b)** Kaplan-Meier survival curves of patient groups from three independent datasets of ER-positive breast tumors [16-18] based on expression patterns of the 14-3-3ζ gene signature. **(c)** Subtype classification of tumors with high expression of the signature genes, based on the five breast cancer molecular subtypes. Data were derived from three independent datasets of ER-positive breast tumors in panel a [16-18], as described in Materials and Methods. **(d)** Association of the 14-3-3ζ gene signature (n = 29 genes) with breast cancer clinical-pathological features. Pearson correlation values are shown. Numbers in bold indicate significant correlations, $P < 0.05$. NA, not available.

Tamoxifen selectively upregulates the zeta isoform of 14-3-3 proteins in breast cancer cells

Based on the findings of a clinical breast cancer gene expression signature associated with high 14-3-3ζ and with risk of recurrence, we undertook studies to examine the effect of perturbing 14-3-3ζ levels on gene regulations and phenotypic properties of ER-positive breast cancer cells. Because 14-3-3ζ belongs to a family of highly conserved proteins, we first examined whether tamoxifen affected regulation of the various members of

the 14-3-3 family. Of note, the mRNA level of only the zeta isoform was markedly upregulated by tamoxifen (Figure 3a, $P = 0.0004$), with 14-3-3ζ reaching the maximal mRNA level by 24 hours (Figure 3b) and maximal protein level at 48 to 72 hours after tamoxifen (Figure 3a). Of the other 14-3-3 isoforms, only 14-3-3β showed low but significant ($P = 0.036$) upregulation by tamoxifen. Cotreatment with tamoxifen and the ER antagonist ligand and ER downregulator, ICI 182,780 (ICI), reversed the stimulatory effect of tamoxifen (Figure 3b), indicating the requirement for ER in the upregulation of 14-3-3ζ.

Functional characterization of the effect of 14-3-3ζ knockdown on the phenotypic properties of ER-positive breast cancer cells

To probe the functional roles of 14-3-3ζ in breast cancer aggressiveness and in antiestrogen resistance, we examined the effect of long-term reduction of 14-3-3ζ on cell phenotypic properties by stable expression of interfering short hairpin shRNA in ER-positive MCF7 cells. We subcloned the human U6 promoter [28] into the plasmid vector pRNAtin and five shRNAs targeting the 3'-noncoding region of 14-3-3ζ and a non-targeting control shRNA were designed. Several clones showed 14-3-3ζ reduction, but only two showed a good level of reduction of 14-3-3ζ (reduction by approximately 60% or 70%). We assume this likely reflects our findings, presented in more detail below, that depletion of 14-3-3ζ greatly slows cell growth and induces apoptosis. Hence, cells are unable to survive in the complete absence of this protein.

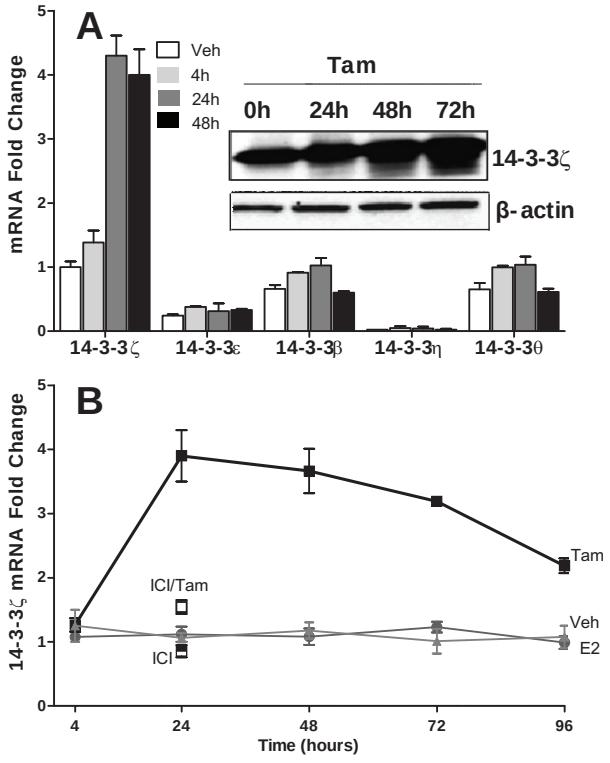


Figure 3 Regulation of 14-3-3 family members in MCF-7 breast cancer cells upon ligand treatment. **(a)** Expression of 14-3-3 isoforms at the indicated times (0, 4, 24, 48 hours) after 1 μM tamoxifen (Tam) treatment. 14-3-3σ is expressed at a very low level and is therefore not shown. Inset shows 14-3-3ζ protein evaluated by western blot after Tam treatment for 0 to 72 hours with β-actin as the loading control. **(b)** Cells were cultured in the presence of vehicle (0.1% ethanol), 1 μM Tam, 10 nM E2, 1 μM ICI 182,780 (ICI) alone or ICI in combination with 10 nM Tam for various times. RNAs were measured by real-time PCR..

We undertook characterization of the two clones showing a downregulation by about 60 to 70%, and found similar trends, so we present data only for the clone showing the greatest 14-3-3ζ depletion (Figure 4a). These cells, referred to as 14-3-3ζ KD, showed 35% and 30% of the parental cell content of 14-3-3ζ at the RNA and protein level, respectively (Figure 4a). This knockdown of 14-3-3ζ did not affect the levels of other 14-3-3 isoforms (data not shown). To validate the specificity of our shRNA knockdown, which was targeted to the 3'-UTR of 14-3-3ζ, we re-expressed 14-3-3ζ cDNA that did not contain the 3'-UTR (denoted KD_R, knockdown and re-expression). Re-expression of 14-3-3ζ in the KD cells substantially restored 14-3-3ζ mRNA and protein (KD_R, Figure 4a).

Cells with downregulation of 14-3-3ζ showed enhanced sensitivity to tamoxifen inhibition of cell viability (Figure 4b). Decreased proliferation of the 14-3-3ζ KD cells was explained by a marked increase of cells in the sub-G1 phase of the cell cycle and a decrease of cells in G1 and G2/M phases (Figure 4c), based on flow cytometric analysis. Moreover, apoptosis was found to be greatly increased with time of tamoxifen treatment in 14-3-3ζ depleted cells compared with control cells (Figure 4d).

14-3-3ζ knockdown impacts FOXM1 and 14-3-3ζ signature genes

Next, we selected several genes from the 14-3-3ζ signature and monitored their levels in cells with stable 14-3-3ζ knockdown. Of note, reduction of 14-3-3ζ was associated with a significant reduction in the expression of signature genes, including BIRC5/Survivin, CDCA8, AURKB, PLK1, BUB1, and CDC25B, and this was reversed by restoration of 14-3-3ζ (Figure 5a). Further, we inspected the cellular level of FOXM1, a transcription factor known to regulate expression of cell cycle genes [29,30], including some of our signature genes. In 14-3-3ζ KD cells, we observed a significant decrease in FOXM1 mRNA and a particularly marked reduction of FOXM1 protein correlating with low levels of 14-3-3ζ (Figure 5a and 5b). Further, re-expression of 14-3-3ζ

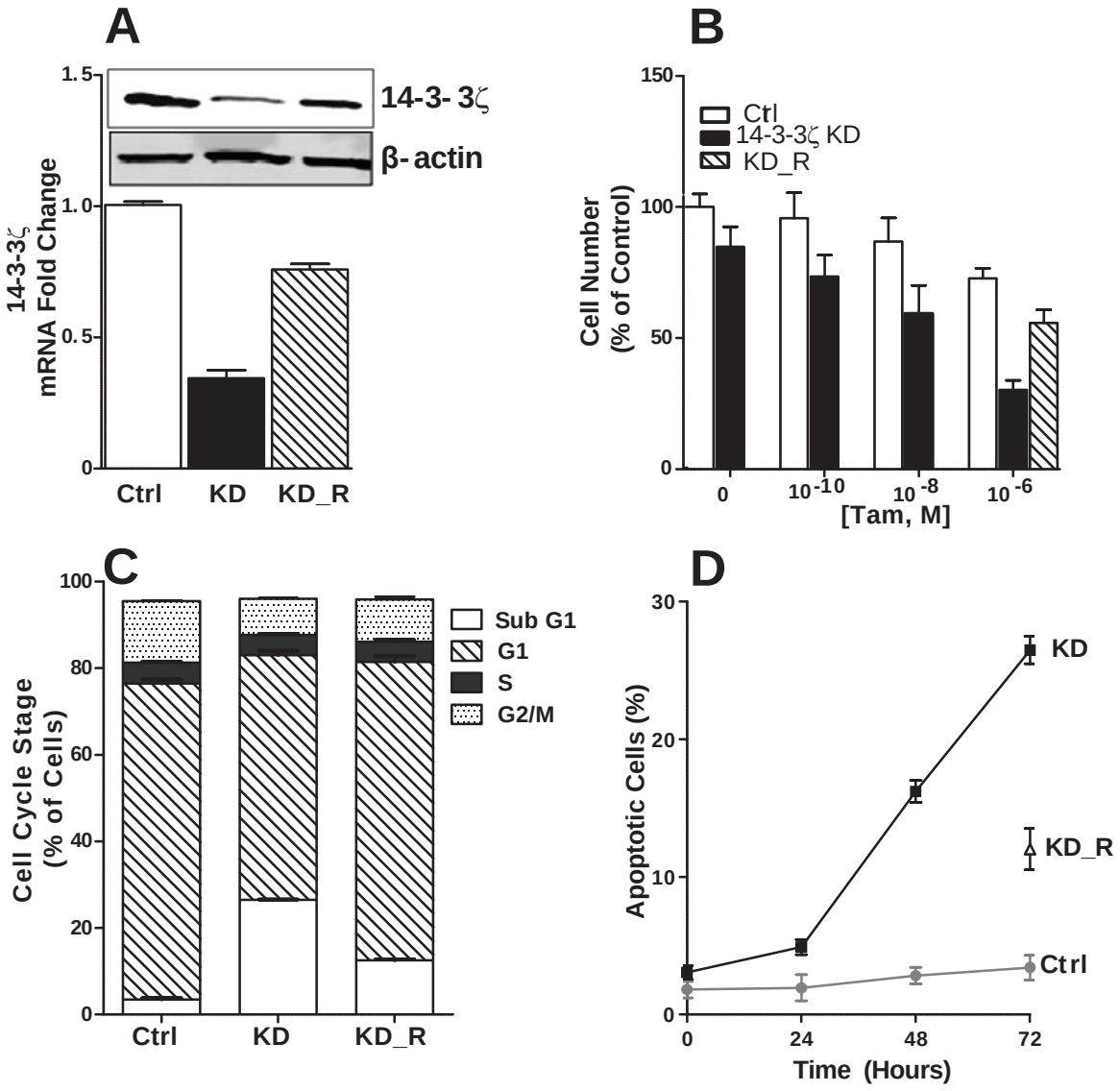


Figure 4 Characterization of the phenotypic properties of MCF-7 cells with knockdown of 14-3-3ζ. (a) 14-3-3ζ RNA and protein levels were evaluated by quantitative PCR and western blot in cells stably expressing control shRNA (Ctrl) or 14-3-3ζ shRNA knockdown (KD) and in KD cells transfected with wild type 14-3-3ζ (KD_R, knockdown and reexpression). **(b)** Cell viability in response to different concentrations of tamoxifen (Tam) for 48 hours for Ctrl or 14-3-3ζ KD or KD_R cells. Cell number for vehicle-treated control cells is set as 100%. **(c)** Percentage of cells in the different cell cycle stages for Ctrl and 14-3-3ζ KD or KD_R cells treated with 1 μM Tam for 72 hours. **(d)** Percentage of apoptotic cells in Ctrl, 14-3-3ζ KD, and KD_R cells treated with 1 μM Tam.

(KD_R cells) substantially restored the level of FOXM1 (Figure 5a and 5b).

By transient knockdown of FOXM1 with siRNA (to 10% of control level, Figure 5c), we observed a marked reduction of AURKB, BIRC5, CDCA8, and CDC25B but little impact on 14-3-3ζ (Figure 5c), indicating that the major regulatory effect of FOXM1 on these genes is downstream of 14-3-3ζ. To explore this further, we treated cells with FOXM1-expressing adenovirus and found that elevation of FOXM1 had no effect on 14-3-3ζ levels in either control or 14-3-3ζ KD cells (Figure 5d), whereas the overexpression of FOXM1 increased expression of the four signature genes and fully abrogated the effect of 14-3-3ζ knockdown (Figure 5d). This pattern of regulation provides support for the regulatory effect of FOXM1 on these genes being downstream of 14-3-3ζ.

Downregulation of 14-3-3ζ in tamoxifen-resistant cells restores sensitivity to the inhibitory effects of antiestrogens

To assess the role of 14-3-3ζ in antiestrogen resistance, we used a tamoxifen-resistant breast cancer cell line

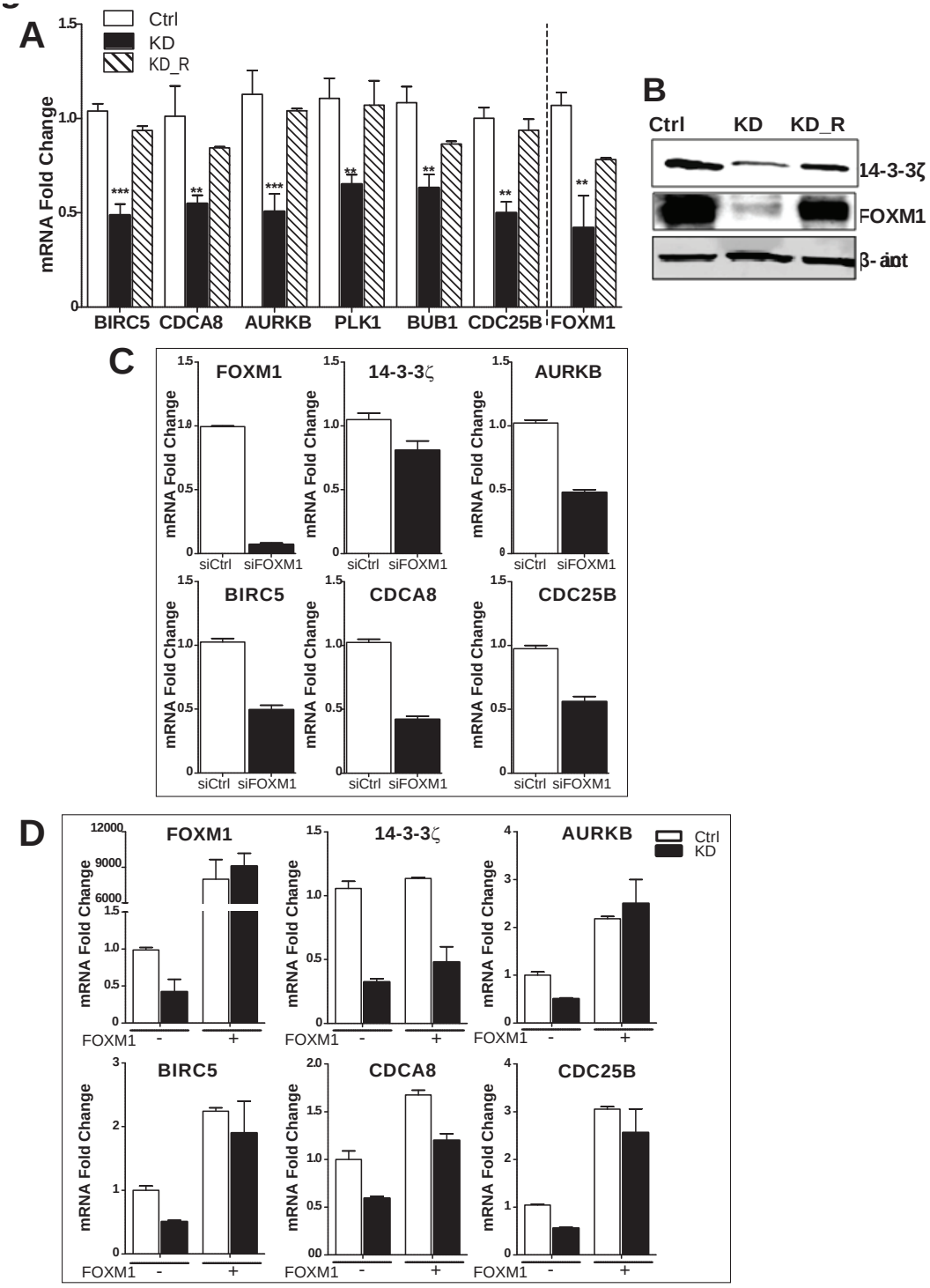


Figure 5 Expression of 14-3-3ζ and associated signature genes in 14-3-3ζ knockdown MCF-7 cells. **(a)** Real-time PCR for six signature genes in control (Ctrl), 14-3-3ζ knockdown (KD) and KD cells with reexpression of 14-3-3ζ (KD_R). Reduced levels of 14-3-3ζ correlated with decreased levels of all investigated genes (black bars; *** $P < 0.001$, ** $P < 0.01$). 14-3-3ζ KD cells with re- expression of 14-3-3ζ (hatched bars) showed gene expression similar to the control (Ctrl) cells. 14-3-3ζ KD was also associated with decreased FOXM1 mRNA that was reversed with reexpression of 14-3-3ζ. **(b)** 14-3-3ζ and FOXM1 protein in Ctrl, 14-3-3ζ KD, and with 14-3-3ζ KD and reexpression. Western blots are shown with actin as loading control. **(c)** Impact of siFOXM1 treatment (black bars) on FOXM1 and expression of 14-3-3ζ and 14-3-3ζ signature genes. **(d)** Re-expression of FOXM1(+FOXM1) in Ctrl and 14-3-3ζ KD cells and its effect on 14-3-3ζ and signature genes. Minus indicates no added FOXM1.

(Tam^R cells) generated in our laboratory [21]. 14-3-3ζ was three times higher in these resistant cells than in the parental MCF7 cells (data not shown), and tamoxifen elicited growth stimulation, rather than growth inhibition, in these cells (Figures 6a and 6b). Knockdown of 14-3-3ζ eliminated tamoxifen stimulation of proliferation and also reduced control cell proliferation (Figure 6b). 14-3-3ζ knockdown also greatly reduced anchorage-independent growth of antiestrogen-resistant cells which grew well in the presence of tamoxifen and raloxifene without 14-3-3ζ knockdown (Figure 6c).

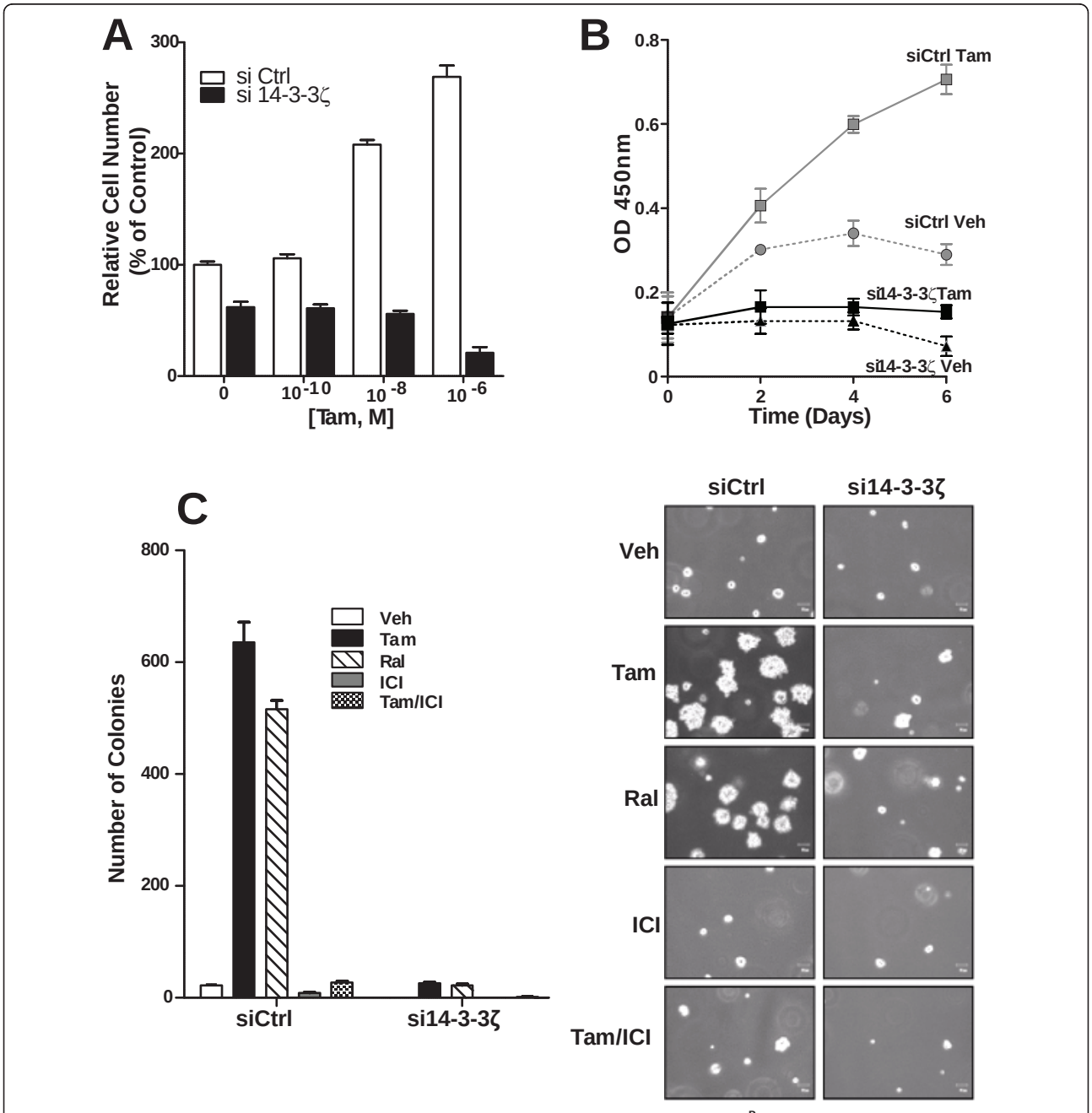


Figure 6 Effects of 14-3-3ζ on viability and colony formation of tamoxifen-resistant (Tam^R) MCF-7 cells. **(a)** Sensitivity to tamoxifen measured as a function of cell viability in response to increasing concentrations of ligand for 96 hours. Vehicle-treated control cells were set at 100%. **(b)** Cell viability monitored over time in Tam^R cells with siCtrl or si14-3-3ζ and treatment with tamoxifen (Tam) for the days indicated. **(c)** Colony formation of Tam^R Ctrl siRNA or 14-3-3ζ siRNA cells in soft agar after 15 days in the presence of vehicle, Tam (1 μM), raloxifene (1 μM), fulvestrant (ICI 182,780) (1 μM) and Tam plus ICI (10 nM and 1 μM). Colonies were stained, counted, and photographed.

With knockdown of 14-3-3ζ in tamoxifen-resistant cells, we observed a downregulation of the 14-3-3ζ signature genes and a marked reduction in FOXM1 (Figure 7a, black bars), and also a suppression of control (veh) cell proliferation and a greatly reduced stimulation of proliferation by tamoxifen (Figure 7b). With FOXM1 overexpression (Figure 7a, hatched bars), expression of 14-3-3ζ signature genes was increased (Figure 7a), and this FOXM1 elevation resulted in an increase in control cell proliferation with only a limited further stimulation by tamoxifen (Figure 7b). When 14-3-3ζ was depleted from cells and FOXM1 was overexpressed, expression of the signature genes was restored to or even increased above the control level (Figure 7a, grey bars), and basal proliferation and stimulation of proliferation by tamoxifen were restored (Figure 7b).

Effect of 14-3-3ζ overexpression or knockdown on markers of hormone resistance

As it is known that enhanced activation of growth factor receptors and downstream kinases can underlie tamoxifen resistance, we examined the impact of 14-3-3ζ status on possible changes in these signaling proteins. We modulated the levels of 14-3-3ζ by adenovirus overexpression or knockdown by RNA interference in tamoxifen-resistant cells, and we monitored over time the status of phosphorylated HER2, EGFR, and downstream signaling kinases AKT and MAPK in cells treated with tamoxifen. Of note, with elevated levels of 14-3-3ζ, the tamoxifen-resistant cells showed enhanced phosphorylation of HER2, EGFR, and MAPK, with lesser impact on pAKT (Figure 8, right). The opposite effects were observed when cells were depleted of 14-3-3ζ, namely suppression of activation of HER2, EGFR, AKT, and MAPK (Figure 8, middle). Hence, 14-3-3ζ plays an important role in modulating the activation status of these key receptors and protein kinases.

Discussion

Endocrine therapies initially provide benefit in many of the approximately 70% of breast cancers that are ER-positive, but the effectiveness of endocrine therapies is often lost with time because resistance to treatment develops. In this study, we show that 14-3-3ζ is a critical factor promoting endocrine resistance. It is upregulated in endocrine-resistant breast cancer and its depletion reverses resistance and restores sensitivity to endocrine treatments.

In probing the functional dimensions of the roles 14-3-3ζ plays in endocrine resistance, we have identified a gene signature associated with high expression of 14-3-3ζ, based on microarray datasets from approximately 400 women with ER-positive breast tumors, and we find that this gene signature is correlated with higher tumor grade, increased metastasis, and risk of early recurrence. Up or downregulating the level of 14-3-3ζ greatly impacted the phenotypic properties of breast cancer cells, including their proliferation, apoptosis, and

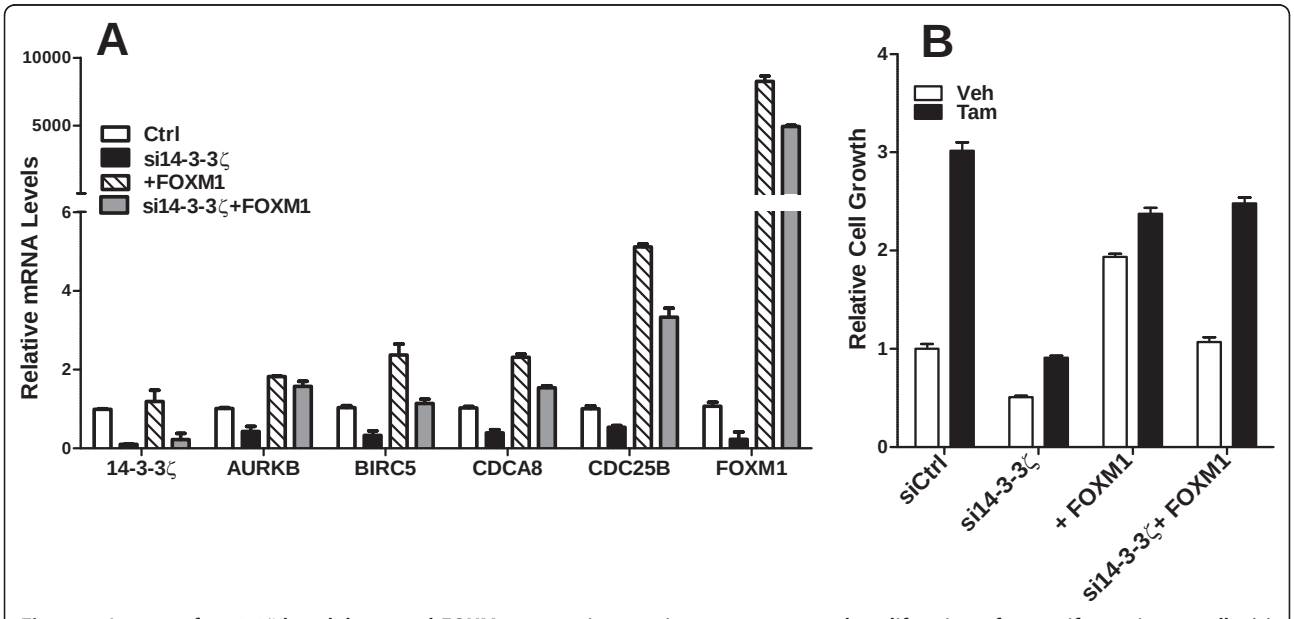


Figure 7 Impact of 14-3-3ζ knockdown and FOXM1 expression on signature genes and proliferation of tamoxifen-resistant cells. **(a)** Expression of 14-3-3ζ, 14-3-3ζ signature genes, and FOXM1 in MCF-7 tamoxifen-resistant cells with siRNA knockdown of 14-3-3ζ and/or overexpression of FOXM1. **(b)** Cell viability monitored after 96 hours of vehicle or tamoxifen (Tam) treatment in tamoxifen-resistant cells with knockdown of 14-3-3ζ and/or overexpression of FOXM1. Control cells transfected with control siRNA were taken as 100%.

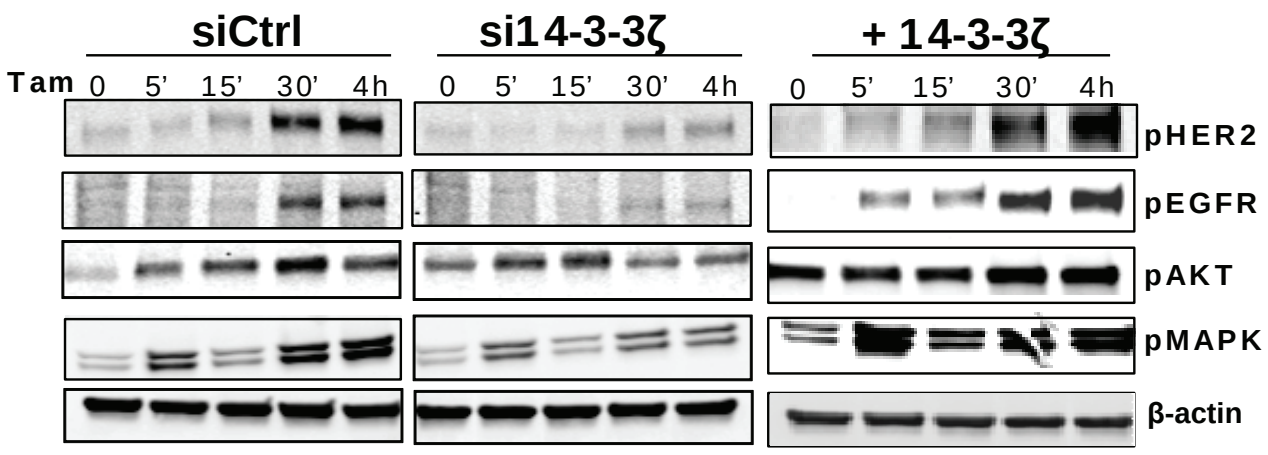


Figure 8 14-3-3ζ status impacts the activation of key signaling proteins. Phosphorylation of human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), AKT/Protein kinase B (AKT/PKB), and mitogen activated protein kinase (MAPK) was investigated by western blot in tamoxifen-resistant cells following siRNA knockdown or overexpression of 14-3-3ζ and treatment with 1 μM tamoxifen (Tam).

endocrine sensitivity. Notably, downregulation of 14-3-3ζ restored sensitivity to endocrine treatments in endocrine-resistant breast cancer cells and reduced the expression of signature genes associated with proliferation and survival, effects that were reversed by re-expression of 14-3-3ζ. Thus, 14-3-3ζ appears to function as a key therapeutic target whose downregulation could improve response to endocrine therapies.

A gene signature and breast cancer molecular subtypes associated with 14-3-3ζ overexpression and poor patient outcome

Using a training set of 67 adjuvant tamoxifen-treated ER-positive breast tumors, we identified, by a supervised analysis, a set of 29 genes that strongly correlated with expression of 14-3-3ζ. By taking advantage of several publicly available large independent breast cancer datasets, we confirmed the ability of the 14-3-3ζ signature to predict clinical outcome. These four large datasets represent a combined total of nearly 400 breast cancer patients with ER-positive tumors. The gene signature was robustly represented by cell cycle-related genes, and factors such as BIRC5/survivin, that have been shown to play important roles in mitosis and to promote cell survival [26,29,31,32].

Our studies are the first to reveal the detrimental role of 14-3-3ζ in endocrine therapy resistance, and they provide a molecular basis for our observation of a poor clinical outcome for women with breast cancers having high expression of 14-3-3ζ. Our findings reveal that 14-3-3ζ is associated with a gene signature rich in genes that encode proteins with central roles in mitosis and the segregation of chromosomes during cell division. The enzyme aurora B kinase, which is part of the chromosome passenger complex, and the protein kinase BUB1 have through recent studies been documented as essential for accurate chromosome inheritance at mitosis. Aurora B kinase appears to act as a fidelity checkpoint factor for mitosis by reversibly phosphorylating target proteins at the centromere and kinetochore [33-35]. BUB1 phosphorylation of a specific threonine in histone H2A has been implicated in the recruitment of the chromosome passenger complex to centromeres [35]. Survivin, also part of our gene signature, binds to aurora B in the chromosome passenger complex, contributing to events that control the normal segregation of chromosomes during cell division. Alterations in the production of these factors, which we observed as a consequence of upregulation of 14-3-3ζ by tamoxifen and associated with the development of endocrine resistance, might thereby also impair proper chromosome segregation and affect cell viability and tumor progression. Indeed, we found that changes in the levels of 14-3-3ζ by knockdown or overexpression had marked effects on cell viability, apoptosis, and on the cell cycle in MCF-7 tamoxifen-resistant cells, and also in ER-positive and HER2-positive BT474 cells based on preliminary studies.

Our studies also uncovered a previously unknown relation between 14-3-3ζ and FOXM1, with 14-3-3ζ playing a crucial role in regulating FOXM1. This was observed in MCF-7 parental and tamoxifen-resistant cells, as shown in this study, and also in ER-positive HER2-positive BT474 breast cancer cells (data not shown). Thus, knockdown of 14-3-3ζ brought about an almost complete loss of cellular FOXM1 protein, which was restored upon re-expression of 14-3-3ζ. Further, the regulation of 14-3-3ζ mitosis-signature genes appears to result from 14-3-3ζ control of FOXM1, because increasing FOXM1 levels in the context of 14-3-3ζ knockdown

effected a parallel restoration of the expression of these genes. Thus, 14-3-3ζ appears to function upstream of FOXM1 in regulating these signature genes. Some of the effects of 14-3-3ζ status on the cell cycle might reflect changes in the cellular level of FoxM1, which is known to regulate genes involved in G2/M, some of which were investigated in this study including BIRC5, AURKB, CDCA8, CDC25B, and PLK1 [29,30].

The majority of ER-positive breast tumors overexpressing 14-3-3ζ were of the luminal B subtype, tumors with a poorer outcome compared with luminal A. Consistent with this, comparative genomic hybridization analyses have indicated that one of the most recurrent alterations in luminal B tumors is gain/amplification of the 8q region that harbors 14-3-3ζ (8q22) [36]. In addition to the prominent association of 14-3-3ζ with ER-positive luminal B tumors, ca. 12% were basal breast cancers, another subtype with a poor prognosis. Collectively, our observations in primary breast tumors and in breast cancer cells *in vitro* provide evidence that the overexpression of 14-3-3ζ and the associated 14-3-3ζ gene signature identify a subgroup of ER-positive tumors most likely to be resistant to endocrine therapies and to show early recurrence. In addition, our studies reveal that 14-3-3ζ expression can also be increased as a consequence of tamoxifen treatment, and therefore, ironically, that tamoxifen itself, through upregulation of 14-3-3ζ, may be contributing to the development of endocrine resistance.

Broad impact of 14-3-3ζ on key cellular activities and signaling pathways

14-3-3ζ status had a great impact on cell signaling pathways and the molecular properties of breast cancer cells. With high 14-3-3ζ, cells showed enhanced activation of EGFR, HER2, MAPK, and AKT, and increased anchorage-dependent and independent growth. These activities were suppressed by downregulation of 14-3-3ζ. Thus, 14-3-3ζ increases signaling through a variety of growth factor receptors and protein kinase pathways, stimulating a more robust and temporally prolonged activation of these pathways to promote survival and anti-apoptotic signaling, and enhance the endocrine resistance of breast cancer cells.

14-3-3ζ is a member of a highly conserved family of 14-3-3 proteins, and it functions as a scaffold or platform that regulates the activity and stability of interacting proteins by binding to their phosphoserine and phosphothreonine motifs. It is noteworthy that 14-3-3ζ is the major form expressed in breast tumors and in ER-positive breast cancer cells, and it was the only 14-3-3 isoform to show high upregulation by tamoxifen. The broad effects of 14-3-3ζ might indeed be expected for a scaffold/adaptor protein that serves as a critical convergence factor in these signaling pathways, having known interactions with EGFR [13], HER2 [13], and PKC [11], as well as additional signaling components such as RAF-1 and β-catenin [12,14]. Our observations now add regulation of FOXM1 as another important aspect of 14-3-3ζ activity in breast cancer and endocrine resistance.

14-3-3ζ as a key marker of endocrine resistance and a therapeutic target for endocrine therapy sensitization

As 14-3-3ζ is overexpressed in breast cancers with a poor prognosis, and its elevated expression is associated with activation of growth factor and mitogenic signaling pathways and with endocrine resistance, our data imply that 14-3-3ζ should serve as a marker of resistance and a key therapeutic target for endocrine therapy sensitization and effective tumor suppression. Resistance to endocrine therapies is associated with enhanced signaling through growth factor receptor and downstream kinase pathways including MAPK and AKT [6,7,9,37-39]. Further, these signaling cascades result in the activation of additional kinases such as polo-like kinase 1 and the cyclin-CDKs, which are part of the 14-3-3ζ gene signature.

Conclusions

In summary, we find 14-3-3ζ to be a key marker for risk of failure on endocrine therapy and show that its elevated expression promoted resistance to endocrine therapies, whereas its downregulation slowed proliferation, enhanced apoptosis, and increased the sensitivity of breast cancer cells to endocrine treatment. From our studies and those of others [10,15,40-43], 14-3-3ζ is emerging as a critical factor that has major impact on multiple forms of cancer therapy, endocrine therapies, and certain chemotherapies as well [44]. Our findings provide new mechanistic insights through definition of a gene signature and molecular phenotype associated with overexpression of 14-3-3ζ that contributes to endocrine resistance. Targeting 14-3-3ζ and the factors it regulates, such as FOXM1, should prove beneficial in delaying the development of endocrine resistance and in reversing resistance, and should allow more effective treatment of patients whose tumors overexpress 14-3-3ζ and are at high risk for disease recurrence.

Abbreviations

ER: estrogen receptor; FDR: false discovery rate; KD: knockdown; PCR: polymerase chain reaction; PI: propidium iodide; SAM: statistical analysis of microarrays; SERD: selective estrogen receptor downregulator; SERM: selective estrogen receptor modulator.

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Authors' contributions

AB conceived and designed the studies, carried out the experiments and data analysis, interpreted the data and wrote drafts of the manuscript. BC carried out some of the experimental studies and data analysis. BSK conceived and designed the studies, analyzed and interpreted the data, and wrote the manuscript. All authors read, made suggestions, and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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